Protoplasma (1998) 204: 114–118

PROTOPLASMA

Thin-alginate-layer technique for protoplast culture of tobacco leaf protoplasts: shoot formation in less than two weeks

Rapid Communication

A. Dovzhenko, U. Bergen, and **H.-U. Koop***

Botanisches Institut, Ludwig-Maximilians-Universität München, Munich

Received September 4, 1998
Accepted September 28, 1998

Summary. Regeneration of plants from protoplasts is regarded a difficult and lengthy procedure which requires well developed skills on the side of the experimenter. Therefore, where alternative procedures for genetic engineering of plants are available, protoplast-based techniques are frequently avoided. Here, we demonstrate, that by our newly developed thin-alginate-layer technique it is possible to regenerate shoots from leaf protoplasts of *Nicotiana tabacum* L. at very high efficiency and very rapidly, with the first shoots appearing within less than two weeks. Root formation is induced on a third medium with first roots being found after only 10 more days of culture.

Keywords: *Nicotiana tabacum*; Protoplast culture; Shoot regeneration; Thin-alginate-layer technique.

Introduction

Plant regeneration from protoplasts was first reported for *Nicotiana tabacum* leaf protoplasts (Takebe et al. 1971) and is now possible with far more than 100 species, including important crop plants like cereals (Fujimura et al. 1985), legumes (Puonti-Kaerlas and Eriksson 1988), and even woody species (Vardi and Spiegel-Roy 1982). Protoplasts are generally cultured in liquid media or embedded in gels of increased osmotic pressure. Following the development of microcolonies with ten to twenty cells, the osmotic pressure is then gradually reduced in a series of culture media differing by not more than 150 mOsm for each step. Macroscopically visible colonies are grown to callus and can then – in many cases again on a different culture medium – be induced to undergo shoot morphogenesis and eventually rooting.

In earlier reports on tobacco, we find values of up to five to six weeks of culture before a colony size of 0.5 to 1 mm is reached (Takebe and Nagata 1984) and the time required for establishing rooted plants is given as three to four months (Gleba et al. 1984). Earlier, in our own experiments, shoot regeneration was observed after a total culture period of four to five weeks (Koop and Schweiger 1985). Here, we report on a novel culture procedure which uses embedding of protoplasts in thin alginate layers (TAL-technique; compare Kuchuk et al. 1998) in combination with further improved culture media and careful observation of physical parameters. The procedure gives very high plating efficiencies and leads to shoot formation in less than two weeks, requiring only two steps and two culture media. Rooting occurs on a third medium after additional 10 days of culture.

Results and discussion

Data presented in the tables refer to experiments performed with *Nicotiana tabacum* cv. petite Havana. Cultivar Wisconsin 38 gave comparable results. All media are presented in Table 1, and the general layout of the experiments is described in Table 2.

Seedlings are germinated on a modified B5 medium (Gamborg et al. 1968) with an increased Mg^{2+} content

*Correspondence and reprints: Botanisches Institut, Ludwig-Maximilians-Universität München, Menzinger Strasse 67, D-80638 Munich, Federal Republic of Germany.

Table 1. Media and solutions for fast regeneration from tobacco leaf protoplasts

Solutions	Media						
	SCN[a]	MMM[b]	Ca-A[c]	AlgA[d]	F-PIN[e]	F-PCN[f]	F-SRN[g]
NH_4NO_3							1600
$(NH)_2SO_4$	134						
KNO_3	2500				1012	1012	1900
$CaCl_2 \cdot 2H_2O$	150		2940		640	640	440
$MgCl_2 \cdot 6H_2O$		1020		1020			
$MgSO_4 \cdot 7H_2O$	1233	1250		1250	370	370	370
KH_2PO_4					170	170	170
$NaH_2PO_4 \cdot H_2O$	150						
Micro-elements	B5				MS	MS	MS
NH_4-succinate					20 mM[h]	20 mM[h]	
Inositol	100				200	200	100
Pyridoxine-HCl	1				2	2	
Thiamine-HCl	10				1	1	1
Ca-panthotenate					2	2	
Biotin					0.02	0.02	
Nicotinic acid	1				2	2	
Sucrose	30 g				ca. 130 g	20 g	30 g
Glucose						ca. 80 g	
Mannitol		ca. 85 g	ca. 85 g	ca. 85 g			30 g
MES[i]		1952	1952	1952	1952	1952	1952
Agar, purified (Sigma)	8 g		10 g				8 g
Alginic acid				12 g			
Benzylaminopurine					1	1	0.1
NAA[j]					0.1	0.1	0.01

Amounts are given as mg/l, unless indicated otherwise. All solutions are adjusted to pH 5.8. The first three media and the last medium are autoclaved, second through sixth medium are adjusted to 550 mOsm.

[a]Shoot culture Nicotiana; modified from B5 medium

[b]Magnesium (20 mM), mannitol, MES (10 mM)

[c]Calcium (20 mM)-agar

[d]Alginic acid, low viscosity, Sigma (St. Louis, Mo., U.S.A.), filter sterilised

[e]Fast protoplast incubation Nicotiana, filter sterilised, vitamin composition after Koop and Schweiger (1985)

[f]Fast protoplast culture Nicotiana, filter sterilised

[g]Fast shoot regeneration Nicotiana

[h]Ammonium succinate added from a 2 M stock solution (filter sterilised) prepared by dissolving 106 g NH_4Cl, 224 g KOH, and 236 g succinic acid to 1 l in double-distilled water

[i]2-(N-morpholino)ethanesulfonic acid

[j]Naphthalene acetic acid

which reduces the appearance of yellow patches on leaves, observed with the original recipe. Seedling roots are removed immediately after germination and seedlings are transferred individually to fresh jars containing the same medium. Leaves for protoplast isolation are best taken from shoot cultures (culture conditions: 25 °C, 16 h light, 0.5–1 W/m^2, Osram L85 W/25 Universal-White fluorescent lamps) two to three weeks of age. 1 mm stripes are incubated overnight with 0.25% cellulase R10 and 0.25% macerozyme R10 (Yakult, Honsha, Japan) dissolved in medium F-PIN (Table 1). Following standard filtration, flotation, and sedimentation procedures (see Koop et al. 1996) protoplasts are suspended in MMM, an MES-buffered medium containing $MgCl_2$ for ionic and mannitol for osmotic stabilisation, and mixed with an equal volume of alginic acid dissolved in the same medium. The alginate solution (AlgA) is filter sterilised rather than autoclaved: this is difficult, but produces the same gel strength at a lower concentration and has proven superior with protoplasts of sugar beet (Hall et al. 1997). (Note: no significant loss in culture efficiency is observed in tobacco if autoclaved alginate, 24 g/l, is used.) Alginate embedding is performed in thin layers by applying 625 µl of protoplast-alginate mixture to the surface of agar-solidified

Table 2. Protocol for fast regeneration of shoots from tobacco leaf protoplasts

	Culture vessel	Volume of medium	Medium	Day number	Efficiency[a]
Seed germination	720 ml glass jar	120 ml	SCN	–21	95%
Shoot culture	720 ml glass jar	120 ml	SCN	–18	
Enzyme incubation	petri dish (10 cm)	10 ml	F-PIN	–1	
Protoplast isolation	12 ml tubes	10 ml	MMM	0	$1.5 \cdot 10^6$ per leaf
Alginate embedding a	petri dish (10 cm)	15 ml	Ca-A	0	
Alginate embedding b	polypropylene grid	625 µl	MMM/AlgA	0	$4 \cdot 10^4$ per grid
Culture step 1	petri dish (6 cm)	652 µl + 2 ml	F-PCN	0–7	
First divisions				2	6.5%
Second divisions				3	77.8%
Colony formation				7	95.8%
Culture step 2	Magenta vessel GA-7	70 ml	F-SRN	8–21	
First trichomes				13	< 1%
Regenerated shoots				21	> 80%
Transfer for rooting	petri dish (10 cm)	20 ml	MS	21	
First roots				30	14%

[a]Efficiencies were taken from selected experiments with cultivars petite Havana; with Wisconsin 38 shoots appeared on day 14 of culture

Ca^{2+}-medium (Ca-A) and immediately inserting a polypropylene grid (10 × 10 meshes, 2 × 2 mm mesh size; Scrynel PP2000, K. H. Büttner GmbH, Wasserburg, Federal Republic of Germany). The use of a thin alginate layer is one of the key steps in the whole procedure. The rationale behind it is that embedding of cells requires thin layers of gels since thicker layers inhibit diffusion. In "single-cell nurse culture" a gel spacer layer only 0.5 mm thick between single target and feeder cells reduces culture efficiency by 50% (Schäffler and Koop 1990). Polypropylene grids mechanically stabilise the gel layer and thus facilitate transfer of the embedded cells/colonies to different dishes during further steps of culture. The grids simultaneously supply a convenient means of defining the location of individual cells for tracking their development during consecutive culture steps (Fig. 1 A–E). Golds et al. (1992) used a similar culture technique ("film layer technique", with even lower gel thickness) in combination with computer-assisted cell tracking. After solidification on Ca-A, which takes about 30 min, grids are carefully removed and placed upside down into liquid culture medium (F-PCN) for replacement of MMM with the medium by equilibration (two times 10 ml, 30 min each) and then transferred to a new petri dish with 2 ml fresh F-PCN. The most prominent features of F-PIN and F-PCN are the absence NH_4NO_3, the reduced content of KNO_3 and the addition of ammonium succinate. In this medium (same culture conditions as for shoot culture except for darkness for the initial 20 h), a new cell wall is formed within a day, initial divisions are already seen on the second day of culture (Table 2 and Fig. 1 B), and colonies of more than 20 cells are formed within only one week (Table 2 and Fig. 1 E). Grids are then placed on solid F-SRN in Magenta vessels, without the requirement of intermediate media for osmotic pressure reduction. F-SRN is a modified MS medium (Murashige and Skoog 1962) with reduced content of phytohormones and the addition of 3% mannitol to avoid vitrification of regenerating shoots. Here colonies grow rapidly: the colony shown in Fig. 1 F and G increased its diameter from 100 to 190 µm in only one day and, on shoot formation, reached more than 300 µm on the following day (Fig. 1 H). We checked for the presence of shoot primordia by studying the absence or presence of trichomes (Fig. 1 H), which never occur on callus. Note, that the developing shoots also differ from callus by the presence of much smaller cells and by their smooth surface. Shoot initials were regularly found after two weeks of culture with a minimum of 13 days in a number of experiments. Shoot formation from almost every colony occurs within three weeks of culture (Table 2). Shoots are removed from the grids and rooted on hormone-free MS medium within the following two weeks of culture (not shown).

In conclusion, we have combined a novel culture technique (thin alginate layer) with new culture media and careful optimisation of physical parameters such as volume of culture vessel, volume of culture medium, and plating density. Experiments were equally

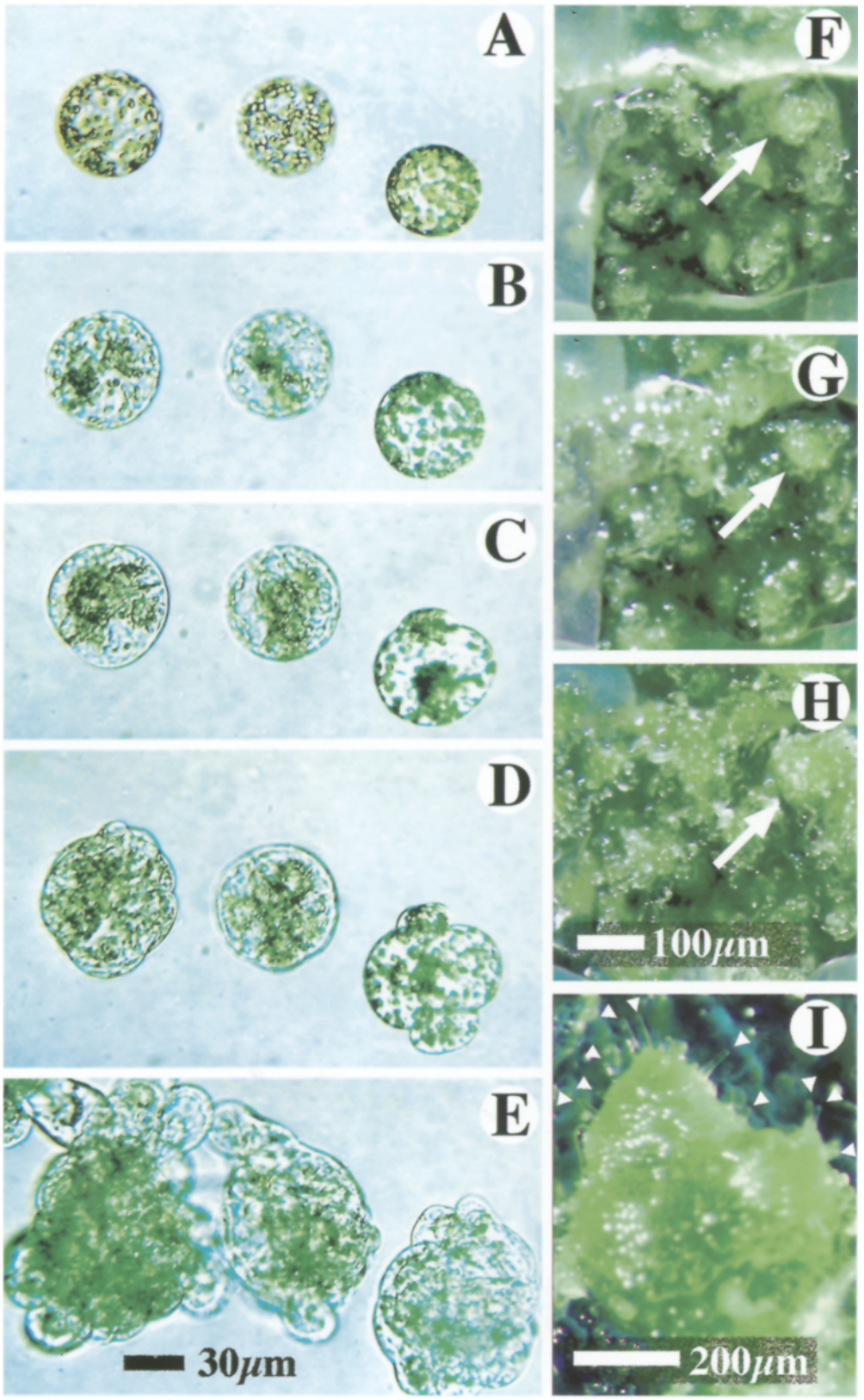

Fig. 1 A–I. Fast shoot regeneration from tobacco leaf protoplasts. **A–E** Development of three randomly selected protoplasts to colonies at days 1 (**A**), 2 (**B**), 3 (**C**), 4 (**D**), and 7 (**E**) of culture. **F–I** Shoot formation from a colony (arrow in **F**, **G** and **H**); a shoot initial (**I**) is recognised by the presence of trichomes (arrowheads). Photographs were taken at days 11 (**F**), 12 (**G**), 13 (**H**), and 14 (**I**)

successful with two cultivars, petite Havana and Wisconsin 38. The highly efficient protocol is simple, it requires only two media for protoplast to shoot regeneration, and intermediate steps for adjustment of osmotic pressure are no longer required. The culture procedure is also extremely rapid: the first shoots appeared in less than two weeks of culture.

Acknowledgements

This work was supported by Bundesministerium für Ernährung, Landwirtschaft und Forsten, grant 95 NR 048-F, and by Deutsche Forschungsgemeinschaft, grant Ko 632-13/2.

References

Fujimura T, Sakurai M, Negishi T, Hirose A (1985) Regeneration of rice plants from protoplasts. Plant Tissue Cult Lett 2: 74–75

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158

Gleba YY, Sidorov VA, Hoffman F (1984) Mechanical isolation and single-cell culture of isolated protoplasts and somatic hybrid cells. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 1. Academic Press, Orlando, pp 405–414

Golds TJ, Babczinsky J, Rauscher G, Koop HU (1992) Computer-controlled tracking of single cell development in *Nicotiana tabacum* L. and *Hordeum vulgare* L. protoplasts embedded in agarose/alginate films. J Plant Physiol 140: 582–587

Hall RD, Riksen-Bruinsma T, Weyens G, Lefèbre M, Dunwell JM, van Tunen A, Krens FA (1997) Sugar beet guard cell protoplasts demonstrate a remarkable capacitiy for cell division enabling applications in stomatal physiology and molecular breeding. J Exp Bot 28: 255–263

Koop HU, Schweiger HG (1985) Regeneration of plants from individually cultivated protoplasts using an improved microculture system. J Plant Physiol 121: 245–257

– Steinmüller K, Wagner H, Rößler C, Eibl C, Sacher L (1996) Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. Planta 199: 193–201

Kuchuk N, Herrmann RG, Koop HU (1988) Plant regeneration from leaf protoplasts of evening primrose (*Oenothera hookeri*). Plant Cell Rep 17: 601–604

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Puonti-Kaerlas J, Eriksson T (1988) Improved protoplast culture and regeneration of shoots in pea (*Pisum sativum* L.). Plant Cell Rep 7: 242–245

Schäffler E, Koop HU (1990) Single cell nurse culture of tobacco protoplasts: physiological analysis of conditioning factors. J Plant Physiol 137: 95–101

Takebe I, Nagata T (1984) Isolation and culture of protoplasts: tobacco. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 1. Academic Press, Orlando, pp 328–339

– Labib G, Melchers G (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58: 318–320

Vardi A, Spiegel-Roy P (1982) Plant regeneration from *Citrus* protoplasts: variability in methodological requirements among *C. limon* cultivars and species. Theor Appl Genet 62: 171–176

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. — Herausgeber: Prof. B. E. S. Gunning, Research School of Biological Sciences, The Australian National University, Canberra City, ACT 2601, Australien. — Redaktion: Sachsenplatz 4–6, A-1201 Wien. — Hersteller: Adolf Holzhausens Nachfolger, Kandlgasse 19–21, A-1070 Wien. — Verlagsort: Wien. — Herstellungsort: Wien. — Printed in Austria