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PROTOPLAST REGENERATION AND ORGANOGENESIS FROM PEA PROTOPLASTS

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SUMMARY

Protoplasts of young Pisum Sativum L. seedlings from 7 different genotypes were isolated and regenerated to the callus stage. Germinating embryos were cultivated with cotyledons removed, thus avoiding intracellular starch accumulation in donor tissue. The first lateral shoots provided a source of homogenous meristematic cells which gave rise to sustained protoplast division and resulted in callus formation within 4 weeks. Root formation occurred on hormone-free medium and shoots developed on medium containing kinetin, 2iP or zeatin in the third subculture, when subcultured in monthly intervals.

Key words: Pisum sativum protoplasts; organogenesis; agarose droplet culture.

INTRODUCTION

In recent years intensive studies of plant protoplasts have led to the development of various adapted regeneration systems in many plant species. Cell division in pea protoplasts isolated from different tissues have been reported (1,2,3,6,8,10). In each case it was possible to obtain the formation of cell clusters and, occasionally, callus. The present communication describes a regeneration system of pea protoplasts which shows rapid callus formation with a reproducibly high plating efficiency, leading to the formation of either shoots (13) or roots on appropriate media.

MATERIALS AND METHODS

Preculture. Dry seeds of seven pea genotypes (Pisum sativum ssp. sativum cv Dippes Gelbe Victoria, cv Birte, cv Belman, cv Bodil, cv Finale, and P. sativum ssp. arvense) were surface-sterilized with 70% ethanol for 30 s followed by a treatment with 5% NaOCl for 5 min. Sterilized seeds were rinsed four times in sterile water and soaked overnight. Testa and cotyledons were then removed and embryo axes cultured on agar (0.7%) solidified hormone-free B5 standard medium (Serva) at pH 5.7 (see Fig. 1a) in a growth chamber at 26° C ± 2° C and a 16h/8h light/dark regime. When the epicotyls reached 1 cm length, the apex was used as source of protoplasts. Remaining embryo axes were kept on medium and protoplasts could be isolated more then ten times from one single axe since formation of adventitious buds continued (Fig. 1b).

Protoplast isolation. Lateral shoots and buds from cotyledon-free pea embryos were cut into slices less than 1 mm and incubated in an enzyme mixture consisting of 3% Macerozyme R-10 (Serva), 4% Cellulase Y-C, 0.2% Pectolyase Y-23 (both Seishin Pharmaceutical) in MES-buffer (3 mM 2-(N-morpholino)-ethanesulfonic acid, 6 mM $CaCl_2$, 0.7 mM NaH_2PO_4, 0.1 M glycine, 0.25 M mannitol) at pH 5.8.

In the first set of experiments, incubation was for 15 h on a discontinuous rotary shaker (15 cycles/5 s at 30 min intervals) at 27° C in the dark. The incubation time could then be reduced to 5 h when the enzyme mixture was desalted with a Sephadex® G-25M PD 10 column (Pharmacia). The enzyme mixture was diluted with buffered seawater (3 mM MES, Winnex® synthetic seasalt from Wiegandt GmbH ad 600 mOsmol/kg) and filtered through a series of sieves (100 μm, 60 μm, 40 μm pore size) removing nondigested tissues. The protoplast suspension was freed of cell debris by centrifugation (350 ×g for 4 min) followed by resuspension in seawater for three times.

Protoplast culture. The final pellet was resuspended in LP* medium (see Table 1) at a plating density of 5×10^4 or 1×10^5 protoplasts/ml using inositol ad 600 mOsmol/kg as osmoticum. Approximately 40 droplets (50 μl aliquots) were placed in a Petri dish (94/16 mm). After the droplets had solidified, 10 ml of liquid medium (without agarose) were added. The Petri dishes were maintained in the dark for one week, followed by slowly increasing the intensity of diffuse light for 2 weeks. At weekly intervals, the surrounding liquid medium was replaced completely and at the same time inositol concentration was reduced gradually (60 gl^{-1}, 45 gl^{-1}, 30 gl^{-1}). Four weeks after the initiation of the protoplast culture, the free floating agarose drops were transferred onto the surface of Gamborgs B5 medium solidified with 0.6% Phytagar (GIBCO) (Fig. 1g). All media used were sterilized by autoclaving.

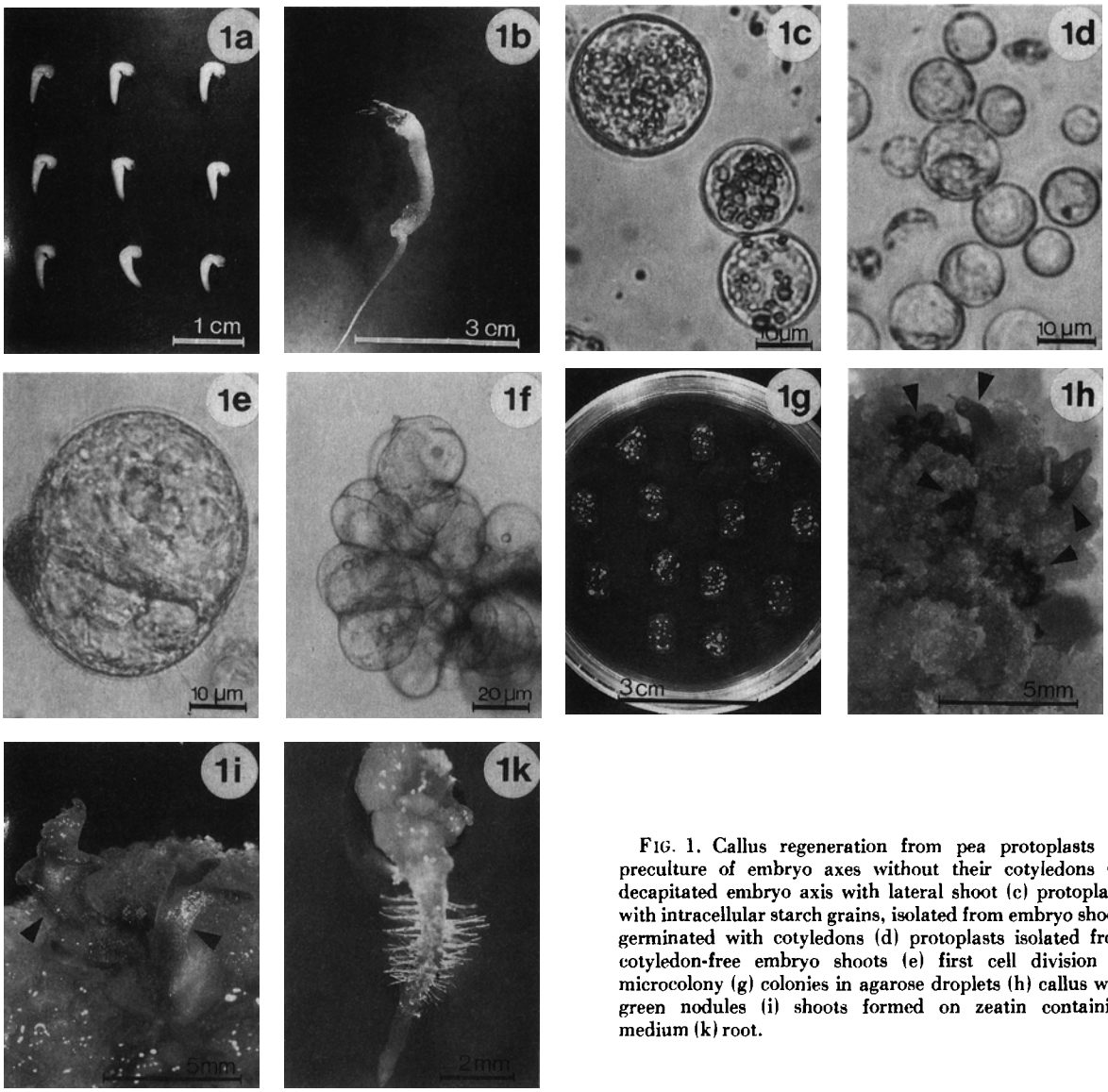

FIG. 1. Callus regeneration from pea protoplasts (a) preculture of embryo axes without their cotyledons (b) decapitated embryo axis with lateral shoot (c) protoplasts with intracellular starch grains, isolated from embryo shoots germinated with cotyledons (d) protoplasts isolated from cotyledon-free embryo shoots (e) first cell division (f) microcolony (g) colonies in agarose droplets (h) callus with green nodules (i) shoots formed on zeatin containing medium (k) root.

Sugar concentrations of liquid media were checked periodically with a handrefractometer (Krüss HRO 32). Plating efficiency was determined after 5 weeks and calculated as the percentage of macroscopically visible colonies obtained from all viable protoplasts at day one.

RESULTS AND DISCUSSION

Previous studies conducted by various groups (see introduction) revealed the importance of tissue source and its physiological state for obtaining cell division in isolated protoplasts. Assuming that highly meristematic tissues are the most promising tissue source, we tested various explants from germinating pea seedlings (leaf, shoot tip, epicotyl, hypocotyl and root tip). In all cases, however, accumulation of starch was observed in isolated protoplasts after one week in culture (Fig. 1c). Similar results were reported by Landgren (1981) with hypocotyl and primary root derived pea protoplasts. Culturing cotyledon-free embryonic axes, we observed no starch accumulation in protoplasts isolated from shoot tips and first lateral shoots (Fig. 1d). Using mannitol or sucrose as osmoticum there were no deleterious effects on protoplast viability, but replaced by glucose (13) we observed 100% dead protoplasts within a few hours (data not shown). The yield of protoplasts was consistantly high (1.7×10^7 protoplasts g^{-1} freshweight) and the viability was determined to be 70–75% (4). Whenever liquid media were used, estimation with the handrefractometer indicated an increasing sugar concentration from initially 3 to 7% within 3 days. In parallel, the pH value dropped to 3.9 immediately. As a result, cell division dramatically decreased in number (13) and supported the budding of nearly all protoplasts.

TABLE 1

MODIFIED LP MEDIUM (LP*)

Inorganics	mgl^{-1}
$CaCl_2$	660*
KH_2PO_4	170*
KNO_3	950*
$MgSO_4 \times 7H_2O$	185*
NH_4NO_3	660*
$MnSO_4 \times 4H_2O$	2.23
H_3BO_3	0.63
Na_2EDTA	18.7
$FeSO_4 \times 7H_2O$	13.9
KI	0.75
$Na_2MoO_4 \times 2H_2O$	0.025
$CuSO_4 \times 5H_2O$	0.0025
$CoCl_2 \times 6H_2O$	0.0025
MES	586*
meso-Inositol	250*
Collidone	500*
Hormones	mgl^{-1}
Picloram	0.2*
Kinetin	0.5*
Organics	mgl^{-1}
Casein hydrolysate	1000*
Folic acid	0.5
Nicotinic acid	2
Pyridoxine	1
Thiamine	5
Glycine	2
Pantothenat	0.87
Sugars	gl^{-1}
Cellobiose	0.25*
Fructose	0.25*
Mannose	0.25*
Rhamnose	0.25*
Ribose	0.25*
Xylose	0.25*
Sucrose	15*
Agarose	3*

Changes to the original composition are marked by *.

As the protoplasts were fixed in agarose droplets, the surrounding medium could be changed completely if required (see also Tan et al. 1987, Gupta et al. 1987). This offered the possibility of removing deleterious compounds and, moreover, of easily stabilizing the sugar concentration and pH value. When shoot tips from entire seedlings were used as the source of protoplasts starch accumulation was not prevented by changing the medium, thus indicating that the cotyledon-free preculture in combination with the medium exchange are the essential steps.

TABLE 2

CELL DIVISION AND PLATING EFFICIENCY FROM DIFFERENT GENOTYPES OF PISUM SATIVUM L.

Genotype	Dividing Cells After 14 Days, %	Plating Efficiency, %
Belman	93–98	6.2
Birte	70–85	1.0
Bodil	83*	1.1
Dippes Gelbe		
Victoria	81–88	3.7
Finale	41*	2.7
P.s.arvense	23–31	not determined

*One experiment, all other values are based on at least five independent experiments.

TABLE 3

SHOOT FORMING CALLIE (%) ON DIFFERENT CYTOKININ MEDIA IN THE P. SATIVUM GENOTYPE BELMAN 90 DAYS AFTER INITIATION

Cytokinins (mgl^{-1})	Calli with Shoots (%)	n
1 kinetin	0	50
1.5 kinetin	2.5	40
2 kinetin	0	40
1 kinetin + 1 ABA	0	80
1.5 kinetin + 1 ABA	0	90
2 kinetin + 1 ABA	0	90
5 2iP	0	90
7.5 2iP	0	90
10 2iP	0	80
5 2iP + 1 ABA	1.1	90
7.5 2iP + 1 ABA	2.0	100
10 2iP + 1 ABA	2.2	90
5 zeatin	0	80
7.5 zeatin	2.0	100
10 zeatin	4.4	90
5 zeatin + 1 ABA	0	90
7.5 zeatin + 1 ABA	5	100
10 zeatin + 1 ABA	10	100

The leaf protoplast medium formulated for pea [LP medium, (2)] as well as other standard media tested [B5, MS (12) or K8p (9)] were not suitable to induce cell division (data not shown), but in LP* viable protoplasts changed their shape within 2 days and cell division began thereafter (Fig. 1e). Plating densities of 10^5 cells ml^{-1} resulted in 30–93% of dividing cells after 14 days (Table 2) and a plating efficiency of 1–6.2%.

When the calli had reached 2 mm they were plated onto LP*, B5 or MS media containing cytokinins. With BAP (1.0 5.0 or 10 mgl^{-1}) calli turned brown within 2 weeks and died, while kinetin, 2iP or zeatin and ±ABA (added after autoclaving) triggered the calli to develop green nodules (Fig. 1h). After the third subculture (subcultured monthly) elongating shoots were visible (Fig. 1i, Table 3), but the shoots were unable to form roots up to now. From callus, roots could be obtained on hormone-free B5 medium within 4 to 5 weeks (Fig. 1k).

Although independently using a comparable protocol as Puonti-Kaerlas et al. (1988), we show an easier method of obtaining protoplasts and microcalli. We also show improved division rates [plating efficiencies not presented in (13)] as well as a more rapid shoot development with identical genotypes.

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