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Mesophyll Cell Protoplasts of Potato

ISOLATION, PROLIFERATION, AND PLANT REGENERATION[1]

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ABSTRACT

Mesophyll cell protoplasts were isolated from potato (*Solanum tuberosum* L. cv. Russet Burbank) leaves and induced to proliferate in culture. Protoplast division was observed only among preparations isolated from plants previously conditioned under short periods of low intensity illumination. Sustained growth and development of protoplast-derived calli (p-calli) occurred when they were maintained on defined media at 24 C under 500 lux lighting. Shoot bud development within p-calli was controlled by a number of factors including light, temperature, basic medium composition, nature and source of phytohormones, the continued presence of an osmoticum, low concentrations of a utilizable carbohydrate, and the developmental stage of the p-callus.

Plant cell protoplasts have advanced considerably as model systems for the study of numerous biochemical and genetic phenomena (4). Especially leaves, but also other plant tissues, offer a ready source of genetically stable cells which may expeditiously be converted into mass populations of protoplasts. When cultured under appropriate conditions, protoplasts isolated directly from tobacco (12) and a limited number of other species (*e.g.* 2, 6, 8, 9) have been induced to reform their cell walls, undergo sustained proliferation, and ultimately redifferentiate whole plants. This feature of protoplast totipotency among the experimental plant species studied thus far suggests a viable new approach toward crop and varietal improvement (3) once similar success is achieved for species of major economic consequence.

The potato, which ranks fourth among world food crops (19), has been recalcitrant in tissue culture. In only a few cases (5, 10, 14, 20) has plant regeneration been achieved from excised tissues other than shoot tips. Recently, however, there has been definite progress toward the development of an *in vitro* regeneration system from single cells. Upadhya (18) cultured protoplasts from potato leaves and obtained calli which differentiated roots but not shoots. Behnke (1) plated cells from dihaploid suspension cultures and reported that "small shoots" emerged in a high percentage of individual calli, although mention was not made of whether whole plants were obtained. In the present study, calli raised from single mesophyll protoplasts of potato were induced to undergo shoot formation and eventually regenerate whole plants. The developmental sequence, while dependent upon the proper balance of phytohormones, was also sensitive to numerous other constituents of the culture medium.

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MATERIALS AND METHODS

Source Plants. The potato (*Solanum tuberosum* L. cv. Russet Burbank) was used throughout this investigation. Propagative source material had originally been freed from potato viruses X (PVX) and S (PVS) by shoot tip culture (17), and was increased thereafter without demonstrable reinfection.

Potato tubers were cut into small (10–15 g) pieces possessing a single "eye" or small sprout. Seed pieces were planted in vermiculite in large (25 cm diameter) plastic pots. Upon emergence, plants were grown to a height of about 30 cm under 15,000 lux of mercury vapor lamp illumination (Sylvania Color-Improved and Brite-White Delux bulbs) with a 12-hr dark period. The environmentally controlled growth room was maintained at a constant temperature of 24 C and a relative humidity of 70 to 75%. Pots were watered daily with approximately 100 ml of a soluble 20-20-20 fertilizer solution (Peters, Inc., Allentown, Pa.) at 1 g/l. Plants were then transferred to a second growth room and maintained from 4 to 10 days at the same temperature and humidity as before but under 6-hr photoperiods of 7,000 lux white fluorescent light.

Isolation of Protoplasts. Well expanded leaflets (9–13 cm in length) were collected from plants conditioned under short photoperiods and surface-sterilized with 0.53% sodium hypochlorite and 70% ethanol (16). Lower leaflet surfaces were gently stroked with a soft nylon brush until they appeared light green (15) and were then cut into squares approximately 2 cm in diameter. Four g tissue were placed in a 500-ml evacuation flask containing 200 ml of medium A (see Table I) without sucrose or agar and then incubated in the dark at 4 C. After 16 to 24 hr, the conditioning medium was replaced with 100 ml of a mixed enzyme solution consisting of 0.3 M sucrose, 0.1 g Macerozyme R-10 (Kinki Yakult Co., Nishinomiya, Japan), 0.5 g Cellulase R-10 (Kinki Yakult Co.), 2 g PVP (mol wt 10,000, Sigma Chemical Co., St. Louis, Mo.), mineral salts of medium A, 0.01 M MES (Sigma Chemical Co.) buffer, pH was adjusted to 5.6 with KOH. Enzyme solutions were then vacuum-infused into the leaf tissue. After 4 hr of incubation at 28 C on a gyratory shaker, 40 rpm, complete digestion of the leaf tissue had occurred and the suspension was poured into a funnel containing four layers of cheesecloth. Protoplasts were collected in Babcock bottles and centrifuged (International model HN-S) at 350*g* for 8 min. During centrifugation, viable protoplasts floated to the meniscus while debris settled to the bottom or remained suspended. Protoplasts were collected, placed in a liquid medium A rinse, and centrifuged as before, but for 5 min. Protoplasts were collected and held in liquid medium A at a density of 6×10^5 cells/ml.

Plating Protoplasts. Medium B (Table I) was poured (20 ml) into 100-mm plastic Petri dishes and allowed to solidify, then 1.5 ml of liquid medium A was pipetted on each plate in order to wet the entire agar surface. Subsequently, all remaining free liquid was removed. Protoplasts were diluted with medium A resulting in a final concentration of 3×10^4 cells/ml. Suspended cells (2

TABLE I. COMPOSITION OF CULTURE MEDIA
These media were from Lam (10). The pH after autoclaving was 5.8.

Constituent	Medium A	Medium B	Medium C	Medium D	Medium E
			mg/l		
NH_4NO_3	0	0	0	1650	1650
KNO_3	190	950	1900	1900	1900
$CaCl_2 \cdot 2H_2O$	44	220	440	440	440
$MgSO_4 \cdot 7H_2O$	37	185	370	370	370
KH_2PO_4	17	85	170	170	170
$Na_2 \cdot EDTA$	3.7	18.5	37.3	37.3	37.3
$FeSO_4 \cdot 7H_2O$	2.8	13.9	27.8	27.8	27.8
H_3BO_3	0.6	3.1	6.2	6.2	6.2
$MnCl_2 \cdot 4H_2O$	2.0	9.9	19.8	19.8	19.8
$ZnSO_4 \cdot 7H_2O$	0.9	4.6	9.2	9.2	9.2
KI	0.08	0.42	0.83	0.83	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.03	0.13	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.003	0.013	0.025	0.025	0.025
$CoSO_4 \cdot 7H_2O$	0.003	0.015	0.030	0.030	0.030
Myo-inositol	10	50	100	100	100
Thiamine · HCl	0.05	0.25	0.5	0.5	0.5
Glycine	0.2	1	2	2	2
Nicotinic acid	0.5	2.5	5	5	5
Pyridoxine · HCl	0.05	0.25	0.5	0.5	0.5
Folic acid	0.05	0.25	0.5	0.5	0.5
Biotin	0.005	0.025	0.05	0.05	0.05
Casein hydrolyzate	100	500	1000	1000	1000
Adenine sulfate	0	0	40	40	40
1-Naphthaleneacetic acid	2	1	0.05	0	0
Indole-3-acetic acid	0	0	0	0.1	0
6-Benzylaminopurine	0.5	0.5	0.5	0	0.5
Zeatin	0	0	0	0.5	0
Gibberellic acid[1]	0	0	0	0	0.1
D-Mannitol	0	0.35M	0.3M	0.2M	0
Sucrose	0.35M	0	15mM	3-15mM	15mM
MES[2]	0	0	5mM	5mM	5mM
Agar[3]	0.5%	1.5%	2.0%	2.0%	1.5%

[1] Gibberellic acid added after autoclaving.
[2] 2-N-Morpholino-ethane sulfonic acid.
[3] Weight:volume, Difco Purified Agar.

ml) were pipetted over the surface of plates containing medium B. Plates containing protoplasts were sealed with Parafilm and incubated at 24 C under continuous illumination of 500 lux.

Plant Regeneration. Initial cell division occurred within 4 to 6 days, followed by continued proliferation into p-calli (protoplast-derived calli). Before p-calli were more than 2 mm in diameter they were transferred to Petri plates containing 20 ml of medium C (Table I), and incubated at 24 C under continuous 5,000 lux illumination for approximately 14 days. Shoot morphogenesis was induced by transferring p-calli from medium C to medium D (Table I), and returning them to the same environmental conditions. P-calli with developed shoots, approximately 1 cm in length, were transferred to medium E (Table I) for final stages of shoot development and root initiation. After the shoots touched the lid, the Petri plates (25 × 100 mm) were incubated at 24 C under dim light (about 200 lux) to promote root initiation. Shoots that were inconveniently slow in rooting were excised from p-calli, and the cut surface dipped in a commercial preparation of Rootone (Amchem Products, Fremont, Calif.). Shoots were then placed in moist sand under high humidity with constant 5,000 lux of illumination. All shoots which had rooted were transferred to peat cups containing fertilized vermiculite and placed in an environmental chamber at 22 C, 70% relative humidity, under 13 hr of 13,000 lux.

RESULTS AND DISCUSSION

Protoplast Isolation and Culture. The environmental and nutritive conditions under which source plants were grown were a critical aspect of protoplast isolation. The exposure of plants to short photoperiods of dim light was especially beneficial for obtaining consistently high yields (*i.e.* 2-3 × 10^6/g tissue) of viable protoplasts. The conditioning of leaf tissue in cold medium A (without sucrose) prior to protoplast isolation generally enhanced the stability of protoplast populations before and after plating.

A variety of media, most of which were modifications of the Murashige and Skoog (11) formulation, were tested for protoplast culture. Of these, the basal composition described by Lam (10) which includes the inorganic salts of Murashige and Skoog and the organic addenda of Nitsch and Nitsch (13) was the most effective when suitably modified. The deletion of NH_4NO_3 and reduction in strength to that shown in medium A (Table I) was most favorable to protoplast viability. When protoplasts in medium A were dispersed over a reservoir of medium B, a plating efficiency of from 20 to 30% was regularly achieved. The bilayer system produced higher plating efficiencies than either medium A or medium B with equivalent final concentrations of sucrose. Each of the organic and phytohormone components shown in Table I for media A and B were essential for optimum protoplast proliferation.

Shoot Morphogenesis. The general sequence of plant development from mesophyll protoplasts of potato is illustrated in Figures 1 to 5. Shoot bud primordia first became identifiable as dark green nodules located at the base of either small (1-2 mm) or larger (up to 15 mm diameter) calli in direct contact with the agar surface. This finding was in contrast to root initials which developed away from the points of contact. Once organized, the shoot bud (when all other conditions were optimal) continued development into a small green shoot with primordial leaves. Multiple shoots emerged from some p-calli, but more frequently, either one or two shoots became fully differentiated. As shoots elongated, they became pubescent and differentiated fully formed leaves.

Of the basic media tested which were supportive of both proliferative growth and of root morphogenesis, only the basal composition of the Lam medium with specific additions and deletions (see Table I, medium D) would also promote shoot morphogenesis. Two features of this medium were apparently responsible for its superiority: the presence of casein hydrolysate, and the organic addenda of Nitsch and Nitsch (13). The omission of the casamino acids from the medium and/or substitution of numerous other organic compounds for those formulated by Nitsch and Nitsch depressed the ability of p-calli to develop shoot buds. When other factors were held constant, these additives permitted a slow pattern of growth among p-calli in the presence of 0.1 mg/l IAA as the sole auxin source. In their absence, IAA was generally insufficient below 1 mg/l with NAA at 0.1 mg/l being far superior for growth. However, IAA at concentrations in excess of 0.1 mg/l or NAA at all concentrations tested was inhibitory to shoot bud development. The modified L medium (medium D) promoted the growth and development of p-calli at exogenous cytokinin (zeatin) and auxin (IAA) concentrations permissive of shoot morphogenesis whereas the other combinations tested did not.

In addition to the organic addenda, casamino acids, and mineral salts, other medium components were also found to influence the development of shoot buds critically. Paramount of these was the concentration of a utilizable carbohydrate source. At phytohormone concentrations permissive of shoot bud development (*i.e.* those of medium D), sucrose levels profoundly influenced the growth pattern of p-calli. At sucrose concentrations of 2% (58 mM) or greater, small (1-2 mm diameter) p-calli grew for only a brief period after which they assumed a brown color and became inhibited. The same effect was recorded between 1 and 2% sucrose (29-58 mM), but the time required for its manifestation varied with the size of the callus. Larger (5-10 mm diameter) p-calli grew for a longer time before tissue regions contacting the medium turned brown. Upper callus regions often remained green and initiated roots but no shoots. However, when the sucrose concentration in medium D was reduced to between 0.2 and 0.5% (6-15 mM), p-calli became deep green and shoot buds emerged in either small (1-2 mm) or larger (5-10 mm) calli.

Another medium ingredient which played a significant role in

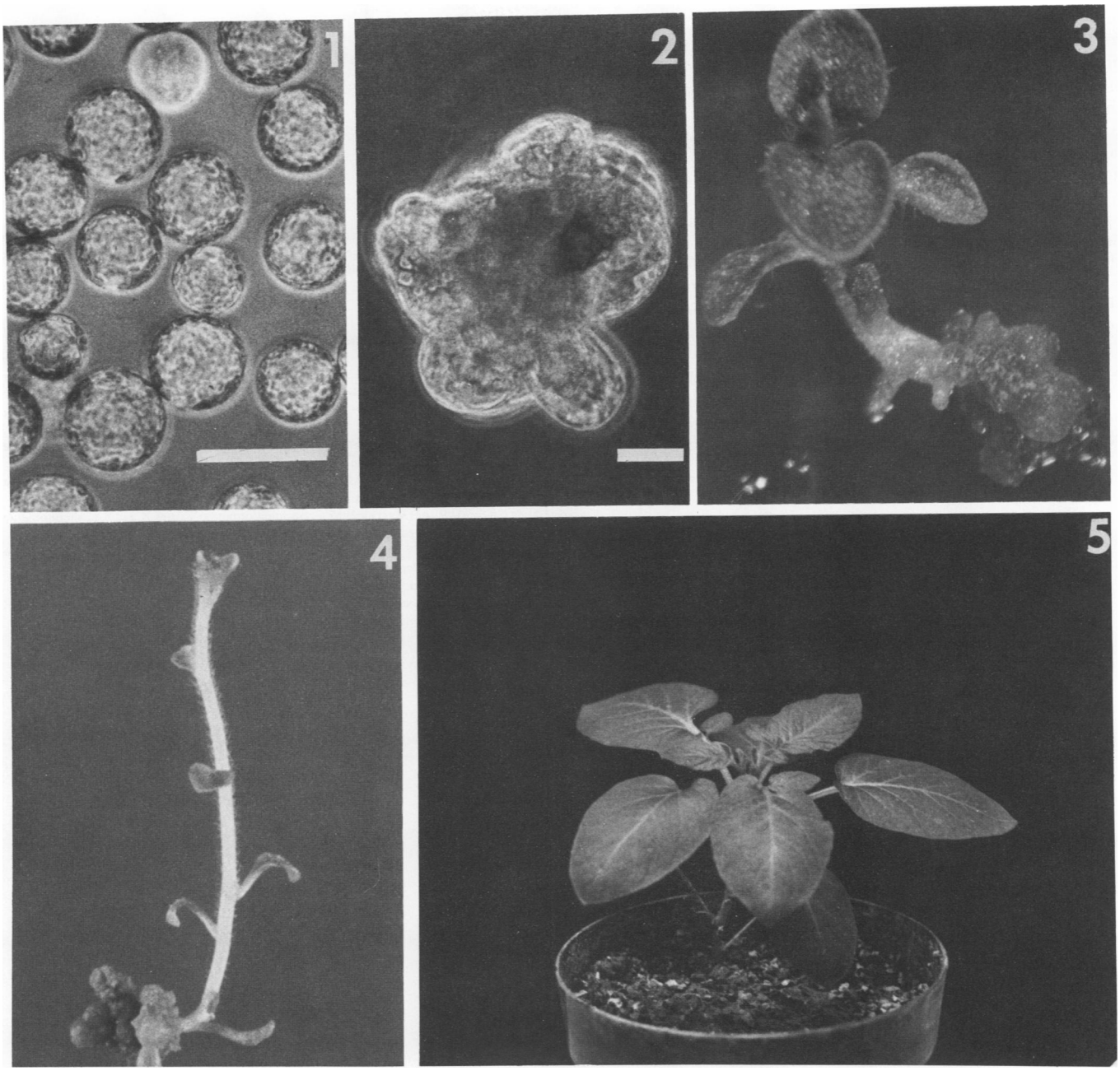

FIG. 1. Protoplasts isolated from leaflets of Russet Burbank potato plants. Scale bar equals 30 μm.
FIG. 2. Small callus derived from a single protoplast. Scale bar equals 150 μm.
FIG. 3. Shoot, 7 mm tall, regenerated from a p-callus which had been transferred from medium C to medium D.
FIG. 4. Elongating shoot, 24 mm tall, which had been transferred from medium D to medium E.
FIG. 5. Fully developed potato plant, 10 cm tall, regenerated from a single protoplast.

the process of shoot morphogenesis was D-mannitol, the optimal concentrations being 0.2 to 0.3 M. On medium D, D-mannitol concentrations below 0.2 M resulted in progressively lower frequencies of shoot bud development with few at 0.05 M D-mannitol or below. In one experiment, 61% of p-calli differentiated shoots on medium D with 0.2 M D-mannitol, but only 2% produced shoots on the same medium with 0.05 M D-mannitol. Equal molar concentrations of *myo*-inositol were successfully substituted for D-mannitol suggesting the possibility of an osmotic effect.

Light intensity and temperature were also critical factors in potato shoot morphogenesis. Intense greening of p-calli occurred at light intensities in excess of 4,000 lux and this was correlated with the potential of p-calli to initiate shoot buds. Little difference was observed between 16-hr photoperiods and continuous light provided that the intensity was sufficient. Temperatures in excess of 28 C depressed growth and development, and this was particularly apparent among p-calli 1 mm in diameter or less when they were first transferred to medium D. Shoot bud development was increasingly inhibited above 24 C. In the vicinity of 28 C, p-calli continued a slow pattern of growth on medium D, but normally did not proceed with the development of shoot buds unless the temperature was reduced.

Over-all, the attainment of shoot morphogenesis in calli raised from mesophyll protoplasts of potato was dependent upon a precise definition of the nutritional, osmotic, and hormonal requirements in concert with light and temperature. Conceptually, potato seems to differ only with regard to specifics from

the other species studied thus far. But arriving at the optimum for each of the possible categories can be a tedious process and nutritional medium components although sometimes described as unnecessary (7) played a key role in determining whether shoot bud morphogenesis would occur.

Shoot Initiation from Tuber Tissue. To ascertain whether or not the cultural conditions found to be determinative for shoot morphogenesis in p-calli were also appropriate for other tissue types, preliminary attempts were made to stimulate shoot development in excised tuber tissue. Potato (cv. Russet Burbank) tubers were surface-sterilized for 60 min in 1.5% sodium hypochlorite, rinsed in sterile H_2O, and cut in half. Tuber plugs, 4 mm in diameter and 10 mm long, were taken with a cork borer and placed on medium C to stimulate a controlled callus development. In 3 to 4 weeks at 24 C under continuous 5,000 lux illumination, compact and light green callus developed among portions of tuber pieces in direct contact with the agar. The tuber plugs were then transferred to medium D and positioned as they were on medium C. Shoots began to emerge within 4 weeks and developed in manner akin to those from p-calli.

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