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Long-term effect of electroporation on enhancement of growth and plant regeneration of colt cherry (*Prunus avium* × *pseudocerasus*) protoplasts

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ABSTRACT

Electric pulses applied to Colt cherry protoplasts enhanced the long-term growth and plant regeneration of protoplast-derived tissues. Protoplasts isolated from long-term cultured tissues derived from electroporated protoplasts retained the ability to enter division in culture earlier and with a higher frequency of plant regeneration than untreated cell suspension protoplasts.

ABBREVIATIONS

BAP, 6-benzylaminopurine; GA_3, gibberellic acid; IBA, 4-indole-3yl-butyric acid; MES, 2-N-morpholinoethane sulphonic acid; MS, Murashige and Skoog (1962); NAA, α-naphthaleneacetic acid; PE, plating efficiency; Z, zeatin.

INTRODUCTION

Electroporation is now a routine technique for introducing DNA into plant protoplasts (Fromm et al. 1986; Hashimoto et al. 1985; Langridge et al. 1985; Shillito et al. 1985). In addition, electroporation has recently been shown to enhance growth of cultured animal tissues (Byus et al. 1987) and undifferentiated plant protoplast-derived cells (Rech et al. 1987). Additionally, enhanced plant regeneration has been demonstrated from tissues derived from electrically stimulated protoplasts of the two woody species Colt cherry (Prunus avium x pseudocerasus; Ochatt et al. 1988) and woody nightshade (Solanum dulcamara L; Chand et al. 1988). Subsequent experiments have demonstrated that, in both these cases, electromanipulation of isolated protoplasts stimulated DNA synthesis (Rech et al. 1988).

This paper reports that electroporation has a long-term effect on protoplast growth and plant regeneration. Colt cherry protoplasts isolated from a long-term cell suspension established from electroporated protoplasts, retained the enhanced growth and plant regeneration exhibited by the original electroporated protoplasts, even though the protoplasts from the established cell suspension were not themselves electroporated.

MATERIALS AND METHODS

Protoplasts were isolated enzymatically from cell suspension cultures of Colt cherry (Prunus avium x pseudocerasus) (Ochatt et al. 1987) and were resuspended, at 2.0×10^6/ml, in 5.0 mM MES-based buffer containing 6.0 mM $MgCl_2$ and 0.7 M mannitol. Four hundred μl samples of this protoplast suspension were given 3 successive exponential DC pulses, at 10 sec intervals, each for 30 μsec at 250 V/cm in the chamber of an electroporator (DIA-LOG, G.m.b.H., 4 Düsseldorf 13, FRG) as detailed previously (Rech et al. 1987).

Initiation of a cell suspension from electroporated protoplasts

Electroporated protoplasts were cultured in MS medium with 1.0 mg/l NAA, 0.25 mg/l BAP, 0.5 mg/l Z and 9% w/v mannitol (Ochatt et al. 1987). Three months later, a cell suspension was initiated by transferring tissue derived from electroporated protoplasts to MS liquid medium with 2.0 mg/l NAA and 0.5 mg/l BAP (designated MSP1). Cultures were maintained on a rotary shaker (90 rpm 25°C, continuous daylight fluorescent illumination of 1000 lux), with subculture every 18 days. This cell suspension, designated ECS, was used as the source of protoplasts for all experiments on the long-term effects of electroporation on growth.

Protoplast isolation from an established cell suspension (ECS)

Protoplasts from the ECS suspension were isolated over 16 successive subcultures and 14 days after each transfer when the cells were in exponential growth. Such protoplasts were cultured alongside non-electroporated (NECS) or recently electroporated (RECS) protoplasts from a control cell suspension culture, isolated during exponential growth 21 days after subculture (Ochatt et al. 1987), for comparison of growth and plant regeneration ability. The control cell suspension was initiated from root callus at the same time as the ECS suspensions.

All protoplasts were cultured in MS medium with 1.0 mg/l NAA, 0.25 mg/l BAP, 0.5 mg/l Z and 9% w/v mannitol. The osmolality of this medium was reduced by mixing the protoplast culture medium with mannitol-free medium in the ratios of 3:1, 2:1 and 1:1 (v/v) when the protoplast-derived colonies had reached the 10, 20 and 30 cell stages (Ochatt et al. 1987). The time in culture required for the onset of protoplast division was assessed using at least 10 replicates for each protoplast type. The PE (defined as the number of dividing protoplasts expressed as a percentage of the total number of protoplasts) at day 10 of culture, and the number of cells per colony, examined at 10-day intervals during the first 30 days of culture, were recorded for all protoplast types. The number of colonies

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(1.0 - 2.0 mm in diam.) in each treatment was determined after 30 days of culture. At this time, colonies were transferred for further growth to agar solidified (0.6% w/v; Sigma) MSP1 medium.

Plant regeneration

After 3 subcultures (each of 2 weeks) on MSP1 medium, plant regeneration was assessed by transferring protoplast-derived calli to MS-based medium supplemented with 0.05 mg/l NAA, 5.0 mg/l BAP, 0.05 mg/l Z and 100 mg/l casein hydrolysate. Cultures were maintained under continuous daylight fluorescent illumination (1000 lux; 25°C; Ochatt et al. 1988). Regenerated buds were grown for 4 weeks on MS medium with 0.1 mg/l IBA, 1.0 mg/l BAP and 0.1 mg/l GA_3, or rooted directly on half-strength MS medium with 0.5 mg/l NAA (Ochatt and Caso 1984).

The number of regenerating calli and the number of regenerated shoot buds per callus were recorded. All assessments included at least 20 replicates for each protoplast type.

RESULTS

Examination of Colt cherry protoplast cultures, to the microcallus stage during the first 30 days of culture, showed that the enhancement of growth resulting from electroporation of isolated protoplasts was sustained even when protoplasts were isolated from long-term cultured tissues derived from electro-treated protoplasts (ECS). Cultured ECS protoplasts entered division earlier than untreated (NECS) protoplasts, but later than those that had been recently electroporated (RECS protoplasts) (Table 1).

Table 1. Number of days to the first division in culture for ECS, RECS and NECS protoplasts of Colt cherry.

Subculture number	ECS	RECS	NECS
1	8	5	14
2	7	4	15
3	7	4	15
4	6	4	14
5	7	3	16
6	9	5	15
7	7	4	14
8	8	-	14
9	7	-	13
10	6	5	17
11	8	4	16
12	7	-	16
13	9	-	15
14	7	4	15
15	8	-	16
16	7	5	14

-, not examined; 10 replicates/treatment

Assessments of the plating efficiency showed that the frequency of division was also higher for ECS protoplasts as compared to NECS protoplasts. The throughput of 1.0-2.0 mm diameter microcalli was higher, after 30 days of culture, from ECS protoplasts (Table 2).

During subsequent culture, there was a consistently higher number of regenerated shoots per callus, with a higher frequency of regeneration from the ECS protoplast-derived tissues as compared to the NECS protoplast-derived calli (Table 2).

Morphological differences were not observed amongst the rooted plants derived from the three protoplast sources; all regenerated plants survived transfer to the glasshouse.

DISCUSSION

The results from these experiments confirm previous observations that electroporation enhances the growth of protoplast-derived cells in culture (Rech et al. 1987), with an associated improvement in plant regeneration from callus derived from such electroporated protoplasts (Chand et al. 1988; Ochatt et al. 1988). In addition, this electro-enhancement of protoplast division and plant regeneration is retained over a number of subcultures, when protoplasts are isolated from an established cell suspension itself derived from electroporated protoplasts.

The response in culture displayed by the electro-treated protoplasts (RECS protoplasts) and by protoplasts isolated from tissues derived from them (ECS protoplasts), as compared with those of the controls (NECS protoplasts), showed that the observed effects of the applied electric pulses cannot be simply the result of a metabolic change induced by the electric pulses applied at the protoplast stage resulting, in turn, in an initial increase of the endogenous hormonal levels. These results, coupled with the increase in DNA synthesis detected for protoplasts of Colt cherry following electroporation (Rech et al. 1988), would suggest that electroporation dictated a more efficient expression of the genes controlling differentiation, as was also observed recently for animal cells (Byus et al. 1987). In this context, it is conceivable that a sustained capacity to take up components of the culture medium, at a higher more selective or more efficient rate, might have been introduced permanently and transmitted to the tissues derived from electroporated protoplasts.

Electroporation, which is currently of widespread application in the genetic transformation of plants, also appears to affect, at least in Colt cherry, basic aspects of plant physiology. These stimulatory effects on protoplast division and plant regeneration may have additional application in somatic hybridisation experiments, where electromanipulation (either of the parental protoplasts prior to fusion or of the heterokaryons) may increase the throughput of somatic hybrid and cybrid plants.

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Table 2. Protoplast responses and plant regeneration frequencies for ECS, RECS and NECS systems.

Subculture	ECS					RECS					NECS				
No.	PE	C/C	MC	RF	S/C	PE	C/C	MC	RF	S/C	PE	C/C	MC	RF	S/C
1	33	5	791	60	7	43	10	1644	75	12	*		196	40	3
2	27	4	804	50	8	44	9	1629	65	9	*		189	35	3
3	30	4	765	60	6	39	10	1681	70	12	*		201	40	1
4	35	3	732	50	7	41	10	1708	70	10	*		213	30	2
5	32	4	787	60	5	46	8	1692	65	12	*		205	30	3
6	29	5	816	70	4	45	9	1677	70	11	*		200	35	2
7	34	4	795	60	6	44	8	1641	75	9	*		194	35	3
8	33	3	776	50	7	-	-	-	-	-	*		199	35	2
9	30	4	763	50	8	-		-	-	-	*		198	30	3
10	28	4	801	60	7	43	9	1724	65	12	*		221	35	3
11	27	5	695	70	5	46	9	1702	75	10	*		206	40	2
12	31	3	773	70	6	-	-	-	-	-	*		202	35	2
13	33	4	780	50	7	-	-	-	-	-	*		209	30	4
14	30	3	822	60	6	46	10	1633	70	12	*		186	35	3
15	37	5	807	60	5	-	-	-	-	-	*		179	30	2
16	29	4	811	60	7	45	9	1647	70	10	*		197	35	2

PE, % plating efficiency; C/C, number of cells per colony after 10 days; MC, number of microcalli produced after 30 days. All data 10 replicates/treatment. RF, regeneration frequency (% of calli exhibiting shoot production); S/C, number of shoots per regenerating callus. All data 20 replicates/treatment.
-, not examined; * occasional first division by day 10.

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