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Plant Protoplast Fusion and Growth of Intergeneric Hybrid Cells*

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Summary. Interspecific and intergeneric fusions of plant protoplasts were induced by polyethylene glycol (PEG) 1540 or 4000. The frequency of heterokaryocyte formation (or rate of fusion) was much higher when PEG was eluted with a high pH-high Ca^{2+} solution or a salt solution than when it was eluted with a protoplast culture medium. The frequency of heterokaryocyte formation was also affected by the types of enzymes used for wall degradation, duration of enzyme incubation and molality of the PEG solutions.

The maximum frequency of heterokaryocyte formation was 23% for *V. hajastana* Grossh.-soybean (*Glycine max* L.) and barley (*Hordeum vulgare* L.)-soybean, 35% for pea (*Pisum sativum* L.)-soybean, 20% for pea-*V. hajastana*, 14% for corn (*Zea mays* L.)-soybean and 10% for *V. villosa* Roth-*V. hajastana*.

40% of the barley-soybean, corn-soybean and pea-soybean heterokaryocytes divided at least once. Some divided many times and formed clusters of up to 100 cells in 2 weeks. The heterokaryocytes of soybean-*V. hajastana*, *V. villosa*-*V. hajastana* also divided. Of the PEG-treated protoplasts of *N. langsdorffii* and *N. glauca* 13.5% developed into tumor-like calli. The morphology of these calli was very much like that of the tumors produced on amphidiploid plants of *N. langsdorffii* × *glauca*.

Nuclear staining indicated that heterokaryocytes of *V. hajastana*-soybean, pea-soybean, corn-soybean and barley-soybean could undergo mitosis. Nuclear divisions in a heterokaryocyte were usually synchronized or almost synchronized. Nuclear fusion and true hybrid formation usually occurred during the first mitotic division after protoplast fusion. A hybrid of barley-soybean in third cell division was observed. The frequency of heterokaryocytes which underwent nuclear fusion has not been determined. Multipole formation and chimeral cell colonies were also observed.

Introduction

Protoplasts from different genera can be induced to fuse by high-molecular-weight polyethylene glycol (PEG; M.W. 1500–6000) (Kao and Michayluk, 1974; Constabel and Kao, 1974). PEG appears to act as a molecular bridge between the surfaces of adjacent protoplasts either directly or indirectly through Ca^{2+}. Fusion presumably results from disturbance and redistribution of electric charges when the PEG molecules are washed away. If this assumption is correct, one should be able to increase the frequency of fusion by increasing the degree of charge disturbance. Since exposure of animal cells and plant protoplasts to solutions containing a high concentration of Ca^{2+} at a high pH has also been shown to induce fusion (Toister and Loyter, 1971, 1973; Keller and Melchers, 1973), an investigation into the combined effects of PEG and high pH-high calcium solutions on fusion is of particular interest. Other factors which also appear to influence protoplast fusion, such as types of enzymes used for cell wall digestion, periods of enzyme treatment and osmolalities of the solutions, were also studied.

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Materials and Methods

Chemicals. Driselase was purchased from Kyowa Kakko Koggo Co., Ohtemachi Tokyo, and used after desalting (Kao *et al.*, 1970). The sources of other chemicals have been given in the previous report (Kao and Michayluk, 1974).

Procedures for Protoplast Isolation and Fusion. Cell suspension cultures of soybean (*Glycine* max L.) (Gamborg *et al.*, 1968) and *Vicia hajastana* (Singh *et al.*, 1970) were maintained in medium 1 (Table 1) under continuous light (fluorescent lamps, 300 lx) at 28° and subcultured every 2 days. Leaves used for protoplast production were derived from greenhouse-grown plants (Kao and Michayluk, 1974). The enzyme solutions used for maceration of tissues and digestion of cell walls are listed in Table 2.

Protoplasts from each species were produced separately. *Vicia* protoplasts from cultured cells were produced by mixing equal volumes of cell suspension with enzyme solution E1 (Table 2). 4 parts of soybean suspension culture were incubated together with 3 parts of one of the enzyme solutions listed in Table 2. Leaves were incubated in a mixture of equal volumes of an enzyme solution (Table 2) and a protoplast culture medium (Table 1). About 1.5 ml of the mixture was put into a 60 × 15 mm Falcon Petri dish to form a thin layer. The dish was sealed with parafilm and incubated at 24°. The dishes were gently shaken for a few seconds each hour (Kao *et al.*, 1973, Kao and Michayluk, 1974). Characteristics of protoplasts from the various plant species and cell cultures are listed in Table 3.

The incubation periods for protoplast production varied from 5 to 24 h. At the end of

Table 1. Cell and protoplast culture media

Compound	Cell culture medium	Protoplast culture medium[d]	
	Medium 1 mg/l	Medium 2 mg/l	Medium 3 mg/l
$NaH_2PO_4 \cdot H_2O$	75	150	150
$KH_2PO_4 \cdot H_2O$	170	—	—
$CaH_4(PO_4)_2 \cdot H_2O$	—	100	50
$CaCl_2 \cdot 2\,H_2O$	295	600	900
KNO_3	2200	2500	2500
NH_4NO_3	600	250	250
$(NH_4)_2SO_4$	67	134	134
$MgSO_4 \cdot 7\,H_2O$	310	250	250
Iron compound (Sequestrene 330Fe)	28	28	28
N—Z-amine[a]	1000	250	250
Micronutrients	as in B5[b]	as in B5	as in B5
Vitamins	as in B5	as in B5	as in B5
2,4-Dichlorophenoxyacetic acid	0.5	0.1	0.1
6-Benzylaminopurine	—	0.5	0.2
NAA	—	—	1.0
Sucrose	25000	—	—
Glucose	—	0.38 M	0.38 M
Xylose	—	250	250
Coconut milk[c]	—	20 ml/l	20 ml/l
pH	5.5	5.7	5.7

[a] Sheffield Chemical, Norwich, N.A., USA.
[b] Gamborg *et al.* (1968).
[c] Heated to 60° for 30 min.
[d] Media 2 and 3 were modified from medium B (Kao and Michayluk, 1974). They are better than Media A and B (Kao and Michayluk, 1974). Medium 2 was especially suitable for soybean, while medium 3 was suitable for *Vicia*. The essential differences between media 2 and 3 are the amount of $CaCl_2$ and hormones.

Table 2. Enzyme solutions for protoplast isolation

Components	Enzyme solution			
	E1	E2	E3	E4
Cellulase (Onozuka P1500)	2%	2%	1%	2%
Driselase	—	—	0.5%	—
Hemicellulase (Rhozyme)	2%	2%	0.5%	2%
Pectinase (Sigma)	1%	1%	0.5%	—
$CaCl_2 \cdot 2\ H_2O$	6 mM	6 mM	6 mM	6 mM
$NaH_2PO_4 \cdot H_2O$	0.7 mM	0.7 mM	0.7 mM	0.7 mM
Sorbitol	350 mM	—	350 mM	—
Mannitol	350 mM	—	350 mM	—
Glucose	—	700 mM	—	700 mM
MES[a]	3 mM	3 mM	3 mM	3 mM
pH	5.7	5.7	5.7	5.7

[a] MES = 2-(N-Morpholino) ethanesulfonic acid.

Table 3. Protoplast species and some of their characters

Species	Source[a]	Characters			
		Diameter (μm)		Vacuolated	Cytoplasmic strands
		Mean	Range		
Soybean (*Glycine max* L.)	SC	30	19–50	highly	rich
Vicia hajastana Grossh.	SC	33	26–53	moderately	rich
V. hajastana	YFEL	31	19–45	moderately	fair
V. villosa Roth	YFEL	25	17–35	moderately	fair
Pea (*Pisum sativum* L.)	YFEL	32	19–43	highly	very poor
Barley (*Hordeum vulgare* L.)	YFEL	36	24–48	highly	very poor
Corn (*Zea mays* L.)	YL	29	16–40	highly	fair
Nicotiana glauca Grah.	YL	—	—	highly	poor
N. langsdorffii Weinm.	YL	—	—	highly	poor

[a] SC = suspension culture; YFEL = young, fully expanded leaves, 1–2 days after the leaflets or young leaf-blades unfolded; YL = young leaves, about 1/4 the length of mature leaves.

the incubation period, the protoplasts were mixed and passed through a 80-μ stainless steel filter. The protoplast suspension was then centrifuged ($50 \times g$, 6 min) and the supernatant discarded. The protoplasts were washed once in either a solution consisting of 3.5 mM $CaCl_2$, 0.7 mM KH_2PO_4 and 0.4 M glucose (Solution C), or in a solution consisting of 3.5 mM $CaCl_2$, 0.7 mM KH_2PO_4 and 0.5 M glucose (Solution D), pH 5.7–5.8 unadjusted. They were then resuspended in one of the above solutions to make a 6% (v/v) suspension.

The PEG solutions used for protoplast fusion are listed in Table 4.

The method of PEG treatment and subsequent protoplast culturing were the same as described before (Kao and Michayluk, 1974), except for the PEG elution procedure. After the protoplasts were incubated in PEG for 40–50 min, two 500-μl aliquots of either a salt solution, a high pH-high calcium solution (Table 5), or a protoplast culture medium (Table 1) were slowly added to the PEG-protoplast preparation at 10-min intervals. After 5 min, further elution was carried out with 1 ml of a protoplast culture medium. Thereafter the protoplasts were washed 5 times at 5 min intervals with a total of 10 ml of the protoplast culture medium.

Table 4. PEG solutions for protoplast fusion

Components	PEG solution		
	P1[a]	P2	P3
PEG 1540	0.33 M	0.33 M	—
PEG 4000	—	—	0.13 M
Glucose	0.1 M	—	0.2 M
$CaCl_2 \cdot 2\ H_2O$	10.5 mM	10.5 mM	10.5 mM
$KH_2PO_4 \cdot H_2O$	0.7 mM	0.7 mM	0.7 mM
pH	5.5	5.5	5.5
Total molality (estimated)	0.44 M	0.34 M	0.34 M

[a] The P1 is the same as the PEG solution F4 described previously (Kao and Michayluk, 1974).

Table 5. Solutions for eluting PEG

Compound	Solution		
	W1[a] (Salt)	W2[b] (High pH-high Ca^{2+})	W3[b] (High pH-high Ca^{2+})
NaCl	128 mM	—	—
$CaCl_2 \cdot 2\ H_2O$	10.5 mM	50 mM	50 mM
Na-glycine buffer	—	50 mM	50 mM
Mannitol	26 mM	—	—
Glucose	27 mM	300 mM	400 mM
pH	6.7	10.5	10.5

[a] Hartmann *et al.*, 1973.
[b] W2 and W3 were freshly prepared, modified from Keller and Melchers (1973).

The frequency of protoplast fusion was examined 16 h after washing. Identification of heterokaryocytes was possible because they contained green plastids from the leaf protoplast(s) and yellowish plastids from the protoplast(s) of the cultured cells, and because cytoplasmic strands across the central vacuole were abundant in the protoplasts from cultured cells but lacking in almost all of the leaf protoplasts. Furthermore, the green heterokaryocytes elongated like their white parents while the green parent protoplasts in the majority of cases remained spherical in shape (Kao and Michayluk, 1974). For observation of nuclear behaviour, the protoplasts and regenerated cells were fixed in acetic acid-alcohol-water and stained with a modified carbol fuchsin (Keller *et al.*, 1973).

Results

A. Fusion of Protoplasts of Vicia, Soybean, Peas, Corn and Barley

1. Fusion of Protoplasts from Cultured Soybean Cells and Vicia Hajastana Leaves. The results in Table 6 indicated that the frequencies of heterokaryocyte formation were consistantly higher when PEG was eluted from the aggregated protoplasts with a high pH-high Ca^{2+} solution than with a protoplast culture medium. The frequencies of heterokaryocyte formation were also increased when a salt solution was used to elute the PEG.

Table 6. The effect of PEG eluting solution on fusion of protoplasts derived from cultured soybean cells and *Vicia hajastana* leaves

Eluting solution[a]	Percentage of heterokaryocytes to total surviving protoplasts and to soybean alone[b,c]				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
	A. Heterokaryocyte formation				
Control (Medium 3, Table 1)	6.9 (12)	5.8 (13)	14.5 (21)	2.4 (5)	2.6 (4)
Salt (W1, Table 5)	13.4 (23)	9.8 (18)	— —	3.1 (7)	5.7 (9)
High pH-high Ca^{2+} (W2, Table 5)[d]	—	—	23.3 (35)	9.9 (22)	24 (53)

Conditions	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
	B. Conditions for protoplast isolation and PEG treatment				
Presence of driselase in enzyme solution (Table 2)	+ (E3)	+ (E3)	+ (E3)	— (E2)	— (E2)
Duration of incubation with enzyme[e]	L	L	L	S	L
Molality of solution for suspending protoplasts (see text)	0.4 (Soln. C)	0.4 (Soln. C)	0.5 (Soln. D)	0.5 (Soln. D)	0.5 (Soln. D)
Molality of PEG solution (Table 4)	0.34 (P3)	0.44 (P1)	0.34 (P2)	0.44 (P1)	0.44 (P1)

[a] After the PEG was eluted with various solutions, the protoplasts were washed and cultured in Medium 3.
[b] Ratio of soybean: *Vicia hajastana* protoplasts in the initial populations was adjusted to approximately 1:1 in all the experiments. Minimum sample size was 500 protoplasts from 2 dishes.
[c] Percentage of heterokaryocytes to soybean protoplasts in parenthesis.
[d] Final pH of the PEG, protoplasts and W2 mixture was 9.5.
[e] L = 8 h at 24°+16 h at 10°; S = 5 h at 24°.

There were significant differences in the results between experiments. The differences could be attributed to three factors: (a) *Enzymes*: the enzyme solution containing Driselase yielded higher percentage of heterokaryocytes than the enzyme solution containing no Driselase (Expts. 1, 2, 3 vs. 4, 5; Table 6A, B). (b) *Length of enzyme incubation*: longer periods of incubation of cells in enzyme solution increased the frequency of heterokaryocyte formation (Expt. 5 vs. 4; Table 6A, B). (c) *Molality of the PEG solution*: a 0.34 M PEG solution resulted in higher frequency of heterokaryocyte formation than a 0.44 M solution (Expts. 1, 3 vs. 2, 4, 5; Table 6A, B, Table 7).

Table 7. Effect of molality of PEG solution on fusion of protoplasts derived from cultured soybean cells and *Vicia hajastana* leaves[a]

Molecular weight	Molality (estimated)		Solution for treating protoplasts	Heterokaryocytes (as % of total surviving protoplasts)
	PEG	Glucose		
PEG 1540 (MW 1300–1600)	0.33	0.1	P1	5.6
PEG 1540	0.33	0	P2	11.8
PEG 4000	0.13	0.2	P3	10.6

[a] Protoplasts were produced by incubating cells in enzyme solutions (E3, Table 2) at 24° for 8 h, then at 10° for 16 h; washed once and suspended in solution D (see text) before PEG treatment. After PEG treatment, all the protoplasts were washed with Medium 3. Minimum sample size was 500 protoplasts from 2 dishes.

2. Fusion of Protoplasts from Cultured Soybean Cells and Pea Leaves. Elution of PEG with high pH-high Ca^{2+} solution also increased the frequencies of pea-soybean heterokaryocytes (Table 8), though the difference between the treated and the control was not as great as in the fusion of soybean and *Vicia* (Table 6) and the increases in relative frequencies of heterokaryocytes resulted from killing of pea protoplasts. However, the size of the heterokaryocytes in the high pH-high Ca^{2+}-treated populations was considerably larger than the control (PEG eluted with Medium 2; Table 1) (Figs. 1–4). This indicated that in the high pH-high Ca^{2+}-treated population almost all the protoplasts in an aggregate had fused together (Fig. 4).

The frequency of heterokaryocyte formation also was influenced by other factors: (a) *Enzymes*: the effect of enzymes was significant. Production of pea protoplasts with enzyme solutions containing Driselase resulted in much higher frequency of heterokaryocyte formation than with enzyme solutions without Driselase (Expt. 5 vs. 4; Table 8). (b) *Length of enzyme incubation*: prolonged incubation of pea protoplasts in the enzyme solution resulted in increased death of pea protoplasts and prolonged incubation did not result in increased heterokaryocyte formation (Expt. 2 vs. 1; Table 8). (c) *Molality of the PEG solution*: a 0.34 M PEG solution produced a higher frequency of heterokaryocytes than a 0.44 M solution (Expts. 3, 4, 5 vs. 1, 2; Table 8).

3. Fusion of Protoplasts from Cultured Soybean Cells and Barley Leaves. Leaf protoplasts of barley were produced readily either by incubating leaf strips in an enzyme solution consisting of Onozuka cellulase, Rhozyme and pectinase (E2, Table 2) or with an enzyme solution consisting of Onozuka cellulase and Rhozyme (E4, Table 2). However, a higher frequency of fusion of barley protoplasts with soybean was obtained when the barley protoplasts were produced in the presence of pectinase. Here again the frequencies of heterokaryocytes were higher when the PEG was eluted with a high pH-high Ca^{2+} solution than when the PEG was eluted with a culture medium (Table 9).

Table 8. The effect of PEG eluting solution on fusion of protoplasts derived from cultured soybean cells and pea leaves

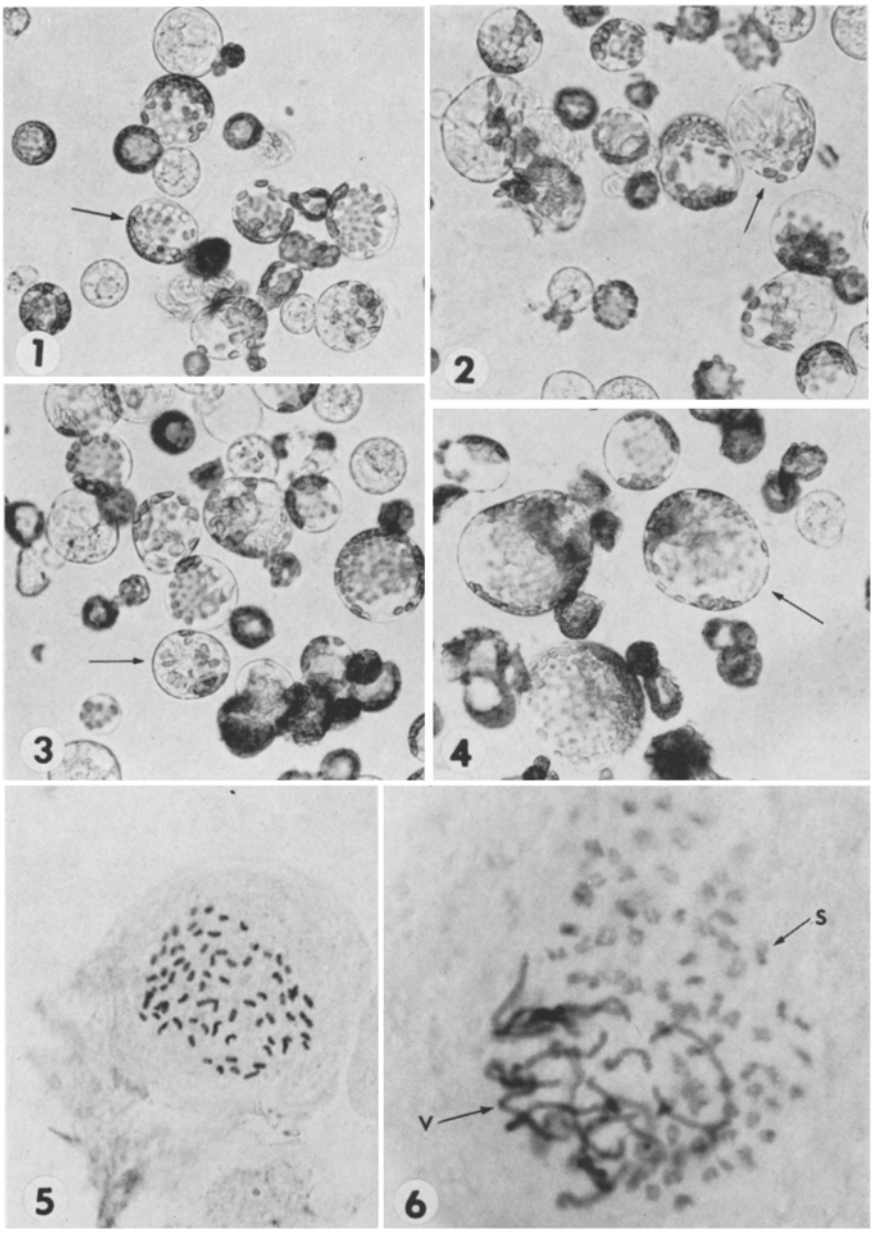
Eluting solution[a]	Heterokaryocytes (as % of total surviving protoplasts and to Soybean alone) [b,c]				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
	A. Heterokaryocyte formation				
Control (Medium 2, Table 1)	—	—	27.5 (44)	17.6 (44)	27.6 (100)
Salt (W1, Table 5)	9.1 (15)	9.4 (11)	—	—	—
High pH-high Ca^{2+} (Table 5)[d]	12.4 (22)	13.7 (16)	31.0 (46)	20.0 (56)	35.3 (91)
Conditions	**Expt. 1**	**Expt. 2**	**Expt. 3**	**Expt. 4**	**Expt. 5**
	B. Conditions for protoplasts isolation and PEG treatment				
Presence of Driselase in enzyme solution (Table2)					
Pea	− (E2)	− (E2)	+ (E3)	− (E1)	− (E3)
Soybean	− (E2)	− (E2)	+ (E3)	+ (E3)	+ (E3
Duration of incubation with enzyme[e]					
Pea	S	L	L	S	S
Soybean	S	L	L	L	L
Molality of solution for suspending protoplasts (see text)	0.5 (Soln. D)	0.5 (Soln. D)	0.5 (Soln. D)	0.5 (Soln. D)	0.5 (Soln. D)
Molality of PEG solution (Table 5)	0.44 (P1)	0.44 (P1)	0.34 (P2)	0.34 (P2)	0.34 (P2)

[a] After the PEG was eluted with various solutions, the protoplasts were washed and cultured in Medium 2.
[b] Ratio of pea: soybean protoplasts in the initial populations was 0.9:1 for Expt. 1 and 2 and 1:1.4 for Expt. 3, 4 and 5. Minimum sample size was 500 protoplasts from 2 dishes.
[c] Percentage of heterokaryocytes to soybean protoplasts in parentheses.
[d] W2 for Expts. 1, 2 and 3; W3 for Expts. 4 and 5. Final pH of the PEG, protoplasts and W2 mixture was 9.5.
[e] L = 8 h at 24°+16 h at 10°; S = 5 h at 24°.

4. Fusion of Protoplasts of Pea with V. hajastana, V. villosa with V. hajastana, and Soybean with Corn. Elution of PEG-treated protoplasts with the salt solution increased frequencies of heterokaryocyte formation between pea (leaf) and *Vicia hajastana* (culture), *V. villosa* (leaf) and *V. hajastana* (culture), and corn (leaf) and soybean (culture) (Table 10).

B. Cell Division in Heterokaryocytes between Vicia, Soybean, Pea, Barley and Corn

Up to 40% of barley-soybean, pea-soybean and corn-soybean heterokaryo-



Figs. 1—4. Degree of protoplast fusion as shown by size of pea-soybean heterokaryocytes (indicated by arrows), 24 h after culturing. Figs. 1 and 3, induced by PEG solution (P2; Table 4); Figs. 2 and 4, induced by PEG (P2) and high pH-high calcium solutions (W3; Table 5). In Fig. 1 and 2, pea protoplasts were produced in an enzyme solution without Driselase (E1; Table 2), while in Figs. 3 and 4 pea protoplasts were produced in an enzyme solution with Driselase (E3) $\times 300$

Fig. 5. An aneutetraploid soybean protoplast in metaphase showing all the soybean chromosomes are small in size, 2 days after culturing $\times 500$

Fig. 6. A *Vicia hajastana*-soybean heterokaryocyte undergoing mitosis, 3 days after culturing. The soybean chromosomes (*s*) are in metaphase, while the *V. hajastana* chromosomes (*v*) are in pro-metaphase. Nuclear divisions are slightly unsynchronized. $\times 1100$

Table 9. Fusion of protoplasts derived from cultured soybean cells and barley leaves[a]

Enzymes for digestion of barley cell wall[b]	Solution for eluting PEG	Heterokaryocytes (as % of total protoplasts and to soybean alone)[c,d]
With pectinase (E2; Table 2)	Medium 2	17.5 (27)
	A high pH-high calcium solution (W3, Table 5)	20.0 (36)
No pectinase (E4; Table 2)	Medium 2	10.6 (19)
	A high pH-high calcium solution (W3, Table 5)	13.6 (36)

[a] Soybean protoplasts were produced in E3 (Table 2). Period of enzyme incubation was 8 h at 24°+16 h at 10° for soybean, 5 h at 24° for barley.
[b] After enzyme treatment, protoplasts were washed once and suspended in solution D. Adhesion and fusion was induced by P1 (Table 4).
[c] Ratio of barley: soybean protoplasts in the initial populations was about 1:1. Minimum sample size was 500 protoplasts from 2 dishes.
[d] Percentage of heterokaryocytes to soybean protoplasts in parenthesis.

cytes formed by the PEG treatment followed by elution with either Ca^{2+} or salt solutions underwent at least one cell division (Table 11). Some formed clusters of up to 100 cells in 2 weeks. The heterokaryocytes of soybean-*Vicia hajasatana* and *Vicia villosa-V. hajastana* as well as protoplasts from cultured cells of soybean and *V. hajastana*, and leaf protoplasts of *V. hajastana* and *V. villosa* also divided. None of the pea, barley, corn protoplasts divided.

Nuclear staining indicated that heterokaryocytes of *V. hajastana*-soybean, pea-soybean, corn-soybean and barley-soybean (Figs. 6–9) could undergo mitosis. Nuclear division in the heterokaryocyte was usually synchronized or almost synchronized. Nuclear fusion and formation of hybrid cells usually occurred during the first mitotic division after protoplast fusion. Premitotic nuclear fusions were rarely observed in the heterokaryocytes as well as in the homokaryocytes; otherwise the nuclear behaviour in heterokaryocytes were very similar to that observed in homokaryocytes (Miller *et al.*, 1970; Kao *et al.*, 1973). A hybrid cell cluster of barley-soybean is shown in Fig. 10. The frequency of heterokaryocytes which underwent nuclear fusion has not been determined. Multipole formation (Fig. 9) and chimeral cell colonies (Fig. 11) were observed. The fate of these hybrid and chimeral cell colonies remain to be determined.

C. Fusion of Nicotiana glauca and N. langsdorffii Protoplasts and Development of the Heterokaryocytes

Cell walls of *N. glauca* and *N. Langsdorffii* leaf cells were removed by incubating very young leaves with the lower epidermis removed in an enzyme solution (E1; Table 2) mixed with an equal amount of Medium 2 (Table 1). Protoplast fusion was induced by treatment with a PEG solution (P1; Table 4) and a salt solution (W1; Table 5).

About 50% of the protoplasts survived after these treatments when cultured in Medium 3 (Table 1). Many divided in 3–4 days and formed cell colonies in

Table 10. Fusion of protoplasts of pea with *V. hajastana*, *V. villosa* with *V. hajastana*, and soybean with corn

Eluting solution[a]	Heterokaryocytes (as % of total surviving protoplasts and to *V. hajastana* or soybean protoplasts)			
	Expt. 1 Pea and *V. hajastana*	Expt. 2 *V. villosa* and *V. hajastana*	Expt. 3 Corn and soybean	Expt. 4 Corn and soybean
	A. Heterokaryocyte formation			
Control (Medium 2; Table 1)	10.0 (11)	6.0 (8)	9.1 (12)	9.5 (11)
Salt (W1; Table 5)	19.5 (21)	10.2 (14)	13.5 (18)	—
High pH-high Ca^{2+} (W3; Table 5)	—	—	—	17.7 (14)

Conditions	Expt. 1	Expt. 2	Expt. 3	Expt. 4
	B. Conditions for protoplast isolation and PEG treatment			
Enzyme incubation (Table 2)	5 h in E1 at 24°	16 h at 24° E1 for *V. hajastana*, E3 for V. *villosa*	5 h at 24° E2 for corn, E3 for soybean	5 h at 24° E2 for corn, 8 h at 24° and 16 h at 10° in E3 for soybean
Molality of solution for suspending protoplasts (see text)[b]	0.5 (Soln. D)	0.5 (Soln. D)	0.5 (Soln. D)	0.5 (Soln. D)
Molality of PEG solution (Table 4)	0.44 (P1)	0.44 (P1)	0.44 (P1)	0.44 (P1)
Ratio of protoplasts in the initial population	1 pea: 1 *Vicia*	1 *V. villosa*: 0.7 *V. hajastana*	1 Corn: 1.2 soybean	1 corn: 3.8 soybean

[a] After the PEG was eluted with various solutions, the protoplasts of pea-*V. hajastana* and corn-soybean were washed and cultured in Medium 2; *V. villosa*-*V. hajastana* in Medium 3.
[b] In Expt. 1, protoplasts were suspended in soln. D after excess enzyme solution was removed; in Expt. 2, 3 and 4 protoplasts were washed once in soln. D and then resuspended is this solution.

Table 11. Frequencies of cell division in heterokaryocytes[a]

Solution for eluting PEG	Cell division in live protoplasts (%)			
	Heterokaryocytes			Soybean protoplasts
	Barley-soybean	Corn-soybean	Pea-soybean	
Control (Medium 2, Table 1)	42	41	38	58
A high pH-high Ca^{2+} solution (Table 5)	40	41	36	53

[a] Other conditions of treatments: Berley-soybean see Table 9 with E2 enzyme solution; corn-soybean see Expt. 4, Table 10; pea-soybean and soybean see Expt. 3, Table 8. Sample size was over 100 protoplasts. Counts were made 6 days after the treatments.

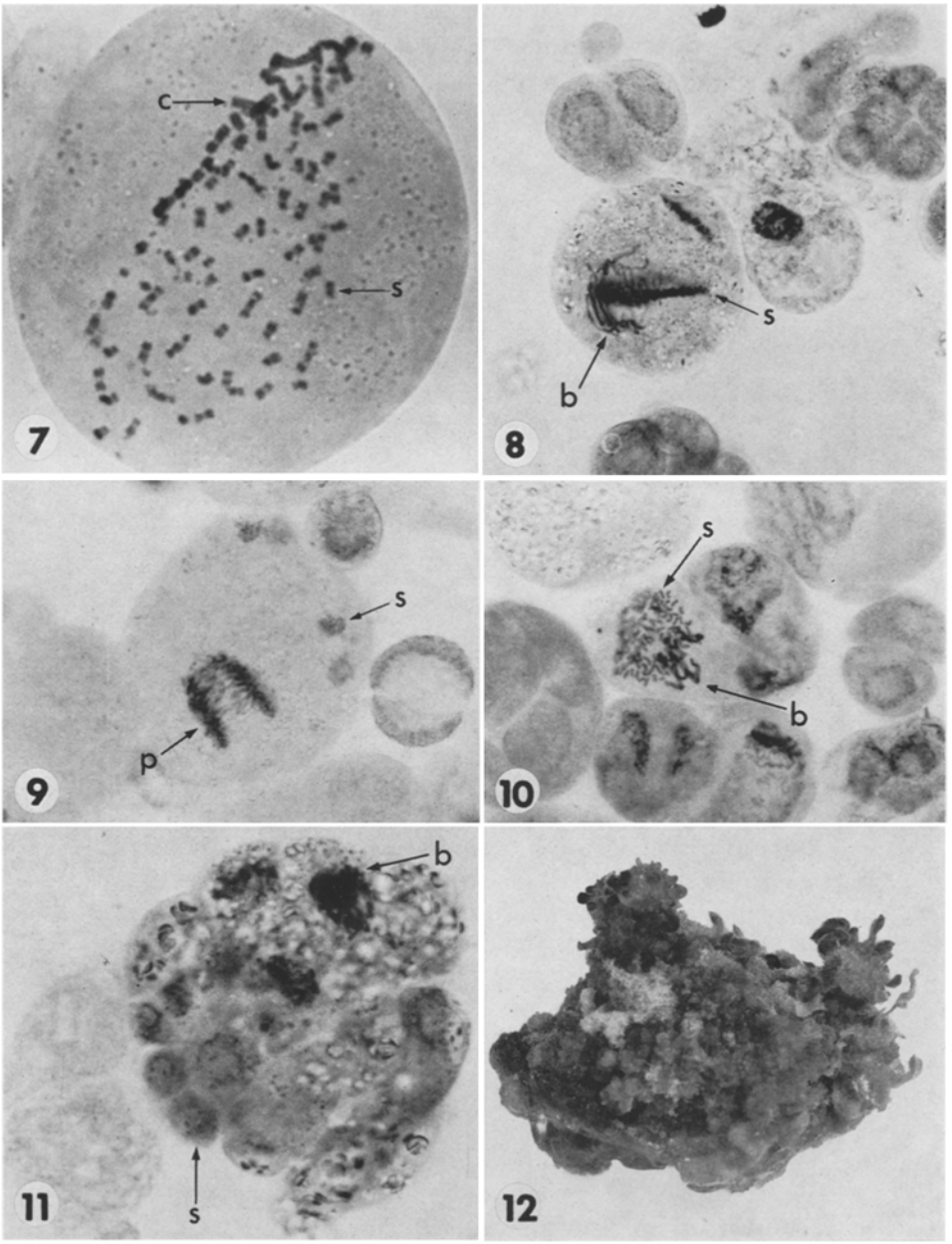


Fig. 7. A corn-soybean heterokaryocyte in metaphase (*c* corn chromosome; *s* soybean chromosome). Synchronized nuclear divisions. 5 days after culturing. ×1100

Fig. 8. A barley-soybean heterokaryocyte, 5 days after culturing. Slightly unsynchronized nuclear divisions (*b* barley chromosome; *s* soybean chromosome). ×500

Fig. 9. A pea-soybean heterokaryocyte, 3 days after culturing, in anaphase, synchronized with multiple poles (*p* pea; *s* soybean). ×500

Fig. 10. A soybean-barley hybrid 5 days after culturing, undergoing third cell division (*s* soybean chromosome; *b* barley chromosome). ×500

Fig. 11. A chimeral cell colony of barley-soybean, 5 days after culturing. Likely originated from a heterokaryocyte (*b* barley nucleus; *s* soybean nucleus). ×500

Fig. 12. A tumor-like callus of *N. glauca-N. langsdorffii* originated from heterokaryotic protoplasts. ×4

2 weeks' time; 238 cell colonies were obtained from *ca.* 1000 PEG -treated live protoplasts. When the small cell colonies were 3–4 weeks old they were transferred onto a modified MS medium (Murashige and Skoog, 1962) consisting of MS mineral salts and sucrose without hormones; the vitamin contents were the same as in B5 medium (Gamborg *et al.*, 1968). These colonies were grown under 16 h light (200 lx)—8 h dark cycles at 24° and 20°, respectively. Within 1 month, 135 of these cell colonies developed into tumor-like calli with indefinite numbers of shoots on each of them. The morphology of these calli was very much like the tumors on the amphidiploid of *N. langsdorffii* × *glauca* (Smith, 1965; Carlson *et al.*, 1972); one callus developed a normal *N. glauca* plantlet and one a plantlet of *N. langsdorffii*; 91 died after being transferred. Thus the frequency of heterokaryocytes in the initially treated population was about 13.5%. This result indicates that PEG could be used successfully in fusion of leaf protoplasts of one species with leaf protoplasts from another species.

Discussion

Plant cell walls are complex structures of polymers. It is unlikely that any single commercial enzyme preparation would have the capacity to remove all the cell-wall materials, although a crude commercial enzyme preparation may contain a number of different wall-degrading enzymes (Karr and Albersheim, 1970). The effect that different enzymes have on protoplast fusion could be due to the degree of cell-wall digestion. The effect that Driselase had on protoplast fusion was obvious. Driselase is a multi-enzyme system produced by a basidiomycyte having a number of zymolytic activities such as cellulase, pectinase, laminarinase, xylanase *etc.* (see "Driselase for protoplast preparation", Kyowa Hakko Kogyo Co., Ohtemachi, Tokyo). The exact components of this enzyme complex are unknown. However, we cannot rule out that this crude enzyme may also contain pronase since it can lyse cell membranes (see "News Fair" No. 3, Kyowa Hakko Kogyo Co.). Slight damage of the membranes may facilitate protoplast fusion (Constabel and Kao, 1974).

If we assume that protoplast membranes would fuse when they have intimate contact (Poste and Allison, 1973), ideally one should fuse protoplasts while in the enzyme solution since true protoplasts free of cell-wall materials may only exist under this condition. However, enzymes trapped between the protoplasts and the glass surface after fusion are very difficult to dilute out, and residual enzymes have a detrimental effect on subsequent cell-wall regeneration and cell division (Kao *et al.*, 1973). Therefore, it is essential to dilute the enzymes out before the protoplasts are treated with PEG. Many protoplasts in the preparations were perhaps spheroplasts rather than true protoplasts. When the spheroplasts are transferred from a solution of a given osmolality to a solution of slightly lower osmolality expansion of the spheroplast occurs. This expansion could result in the exposure of true membranes and thus facilitate fusion. Prolonged incubation of the protoplasts in the enzyme solutions reduced the capacity of the protoplast to regenerate the cell wall; this is perhaps the reason why prolonged incubation of the protoplasts in the enzyme solution increased the frequency of fusion.

Elution of the PEG with the high pH-high Ca^{2+} or salt solution resulted in a much higher rate of fusion than when the PEG was eluted with a culture medium. This indicates that the PEG induced fusion can be enhanced by increasing the degree of charge disturbance. The combination of PEG with high pH-high Ca^{2+} or with salt are especially effective in promoting protoplast fusion when only limited areas of the cell membranes are in contact with each other. This limitation of membrane contact may be due to the small size of the protoplasts or their membrane being partially covered with residual or newly synthesized wall materials.

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