식물학회지 제 30 권 제 4 호
Korean J. Bot. 30(4) : 287-298(1987)

Culture and Fusion of Protoplasts from Potato (*Solanum tuberosum* L.) and Tobacco (*Nicotiana tabacum* L.)

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감자(*Solanum tuberosum* L.)와 담배 (*Nicotiana tabacum* L.)의 원형질체 배양 및 융합

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ABSTRACT

The regenerative capacities of protoplasts isolated from potato (*Solanum tuberosum* L.) tubers and tobacco (*Nicotiana tabacum* L.) mesophyll tissues were examined, and then their intergeneric protoplast fusion was carried out. The potato tuber-derived protoplasts proliferated into the calli some of which showed rudimentary shoot-like structures, which had not been attempted before from tubers, while the tobacco protoplasts were regenerated into the whole plants. Intergeneric protoplast fusion between potato and tobacco was carried out and the heteroplasmic fusion products were formed. The first cell division of some of them was observed after 5 days of culture.

INTRODUCTION

In plants, apart from animals, a single cell has the capacity to regenerate into a whole organism, though it is at present restricted to a relatively few genera. Recently, research has progressed in the production of new hybrids without recourse to conventional breeding methods. One possible approach to achieve it involves the formation of hybrids by fusion of protoplasts from different plant sources (Vasil, 1976; Gamborg *et al.*, 1979; Schieder and Vasil, 1980). High yield of viable protoplasts from tissues and culture of the isolated protoplasts are prerequisite to attempt the above aim of research. However, despite numerous attempts by several workers it has not been possible so far to derive whole plants from protoplasts isolated from the tissues of the economically more important species (Bhojwani *et al.*, 1977; Dale, 1983).

In the case of the potato, which ranks fourth among world food crops (Wade, 1975), plant regeneration from the leaf mesophyll cell protoplasts and the shoot culture-derived protoplasts has been successfully accomplished (Shepard and Totten, 1977; Binding *et al.*, 1978; Thomas, 1981; Kikuta *et al.*, 1983). However, there has been no report as yet that protoplasts isolated from potato tuber tissues as well as other plant tubers were regenerated into the whole plants. We previously reported on the isolation of protoplasts from potato tuber cells (Chung and

Sim, 1986).

In the present study, protoplasts isolated from potato tuber tissues and tobacco leaf mesophyll cells were cultured, respectively, to ensure the regenerative capacities before using them as fusion partners. Protoplast fusion between potato and tobacco was then carried out.

MATERIALS AND METHODS

Plant naterials. Potato (*Solanum tuberosum* L,) tubers used were from cultivars Dejima and Superior which had been stored at 4℃ until use after harvest. They were kindly provided by Horticultural Experiment Station, O.R.D., Suweon, Korea. Tobacco (*Nicotiana tabacum* L. cv. NC2326) seeds, provided by Korea Ginseng Tobacco Research Institute, Suweon, Korea, were sown and were grown under conditions of 15,000 lux of fluorescent light with an 8 hours dark period and a constant temperature of 27℃. Plants were watered with a Hyponex solution at 1g/l. Young but fully expanded leaves were harvested after plant had reached approximately 70 cm tall and had not begun to flower.

Isolation of potato protoplasts. Detailed procedures for protoplast isolation from potato tubers were described previously (Chung and Sim, 1986). After enzyme treatment, the protoplasts were washed three times in the protoplast culture medium (Table 1).

Isolation of tobacco protoplasts. Leaves were washed thoroughly in running water and excess water was removed with a filter paper, then placed in the refrigerator overnight. Then, they were surface-sterilized with 70 % ethanol for 2-3 minutes, dried in a sterile air chamber and the lower epidermis was removed. The exposed leaf areas were cut into strips (1×1 cm) and they were placed in 5 ml of filter-sterilized enzyme solution contained in 60 mm Petri dish. The enzyme solution consisted of 0.7 M mannitol, Onozuka cellulase R-10 (Kinki Yakult Co., Nishinomiya, Japan), Macerozyme R-10 (Kinki Yakult Co.) and mineral salts of protoplast culture medium (Table 1). pH was adjusted to 5.7 with KOH. After incubation at 27℃, the released protoplasts were filtered through a 61 μm stainless steel mesh and recovered in a conical centrifuge tube. They were washed three times in protoplast culture medium by centrifugation at 75 × g for 5 minutes. The number and viability of protoplasts were assessed as described by Chung and Sim (1986).

Culture of potato protoplasts. Protoplasts were suspended in the protoplast culture medium(P-5 medium of Table 1) resulting in a final concentration of 3×10^4 cells/ml. 2 ml of the suspended protoplasts were cultured in 50 mm plastic Petri dishes. Dishes were sealed with Parafilm and incubated in the dark at 27℃. After 3 days of culture, the protoplast suspensions were diluted 1:1 with the medium of reduced mannitol content (0.3M) containing 0.4% agar. The preparations were again distributed as 2 ml aliquots. Every 4 to 5 days fresh medium containing 0.2% agar was added to the developing colonies. Callus colonies reaching the size of 0.8-1.0 mm in diameter were gently transferred onto the agar surface of p-callus medium (Table 1) using a sterile Pasteur pipette or a fine pointed forceps. They were then incubated

Table 1. Composition of culture mediia

Colstituent	Potato[1]		Tobacco				
	Protoplast culture(p-5)	p-callus culture	Protoplast[2] culture	Shoot induction I[3]	Shoot induction II[4]	Shoot induction III[5]	Root induction
	mg/l						
NH_4NO_3	–	1650	825	825	1650	1650	825
KNO_3	950	1900	950	950	1900	1900	950
$CaCl_2 \cdot 2HO$	220	440	220	220	440	440	220
$MgSo_4 \cdot 7H_2O$	185	370	1233	1233	370	370	185
KH_2PO_4	85	170	680	680	170	170	85
$Na_2 \cdot EDTA$	18.63	37.3	37.3	37.3	37.3	37.3	37.3
$FeSO_4 \cdot 7H_2O$	13.93	27.8	27.8	27.8	27.8	27.8	27.8
$MnCl_2 \cdot 4H_2O$	9.9	19.8	19.8	19.8	19.8	19.8	19.8
KI	0.42	0.83	0.83	0.83	0.83	0.83	0.83
$CoSo \cdot 7H_2O$	0.015	0.03	0.03	0.03	0.03	0.03	0.03
$ZnSo \cdot 7H_2O$	4.3	8.6	8.6	8.6	8.6	8.6	8.6
$CuSo_4 \cdot 7H_2O$	0.013	0.025	0.025	0.025	0.025	0.025	0.025
H_3BO_3	3.1	6.2	6.2	6.2	6.2	6.2	6.2
$Na_2MoO4 \cdot 2H_2O$	0.125	0.25	0.25	0.25	0.25	0.25	0.25
Glycine	1	2	2	2	2	2	2
Nicotinic acid	2.5	5	5	5	5	5	5
Pyridoxiine · HCl	0.25	0.5	0.45	0.5	0.5	0.5	0.5
Thiamine · HCl	0.25	0.5	0.5	0.5	0.5	0.5	0.5
Folic acid	0.25	0.5	0.5	0.5	0.5	0.5	0.5
Biotin	0.025	0.05	0.05	0.05	0.05	0.05	0.05
myo-Inositol	50	100	100	100	100	100	100
Casein hydrolyzate	500	1000	–	–		–	–
AII	–	–	–	4	0.5	–	–
NAA	–	–	3	–	–	–	–
2,4-D	2	2	–	–	–	–	–
BAP	–	–	1	1	1	1	–
Kinetiin	–	–	–	2.56	–	–	–
Zeatin	0.5	0.5	–	–	–	–	–
Sucrose	29mM	29mM	29mM	0.3M	58mM	58mM	29mM
Mannitol	0.5M	0M3M	0M7M	–	0.33M	–	–
Agar	0.2%	0.6%	0.6%	0.7%	0.7%	0.7%	0.7%

1) Modified from Lam(1975, 1977) and Shepard and Totten(1977)

2) Modiified from Nagata and Takebe(1971)

3) Modiifiied from Shepard and Totten(1975)

4) Modified from Bourgin *et al.* (1979)

5) Modified from Hayashi and Nakajiima(1984)

pH was adjusted to 5.7 with KOH before autoclaving.

under light of 500 lux (16h day/8h night). After 10 days of culture in this medium, calli were transferred to the fresh medium with a lowered mannitol content (0.25 M) and dishes were placed in a growth chamber with ligh intensity of 2,000 lux at 21℃ for 2 weeks. They were then cultured in the medium containing 0.2 M mannitol under 5,000 lux illumination.

Culture of tobacco protoplasts. Initial step of protoplast culture procedure was similar to that of Nagata and Takebe (1971). Protoplast suspension (1.0×10^5 cells/ml) in protoplast culture medium (Table 1) was mixed gently with an equal volume of melted agar (1.2%) medium which had been kept at 45℃, and 5 ml aliquots werr poured into 60 mm Petri dishes. The dishes were sealed with Parafilm and incubated at 27℃ with a light intensity of 1,500 lux over a photoperiod of 16/8 hours. When the colonies reached the size of 0.3-0.6 mm in diameter, the small agar blocks containing calli were transferred onto the shoot induction medium (Table 1) using a small spatula. Fully developed shoots (about 3 cm tall) were excised and transferred to the root induction medium (Table 1). Shoots produced roots in this medium were transplanted into soil in pots.

Protoplast fusion and culture of heterokaryocytes. Fusion of potato and tobacco protoplasts was performed by similar procedures of Kao(1975, 1981) and Constabel (1984). After enzyme treatment, the isolated protoplasts of each species were washed once with solution D (Table 2)

Table 2. Solutions for protoplast fusion

Compound	Solution		
	D	PEG	W2
Glucose	9g	–	5.4g
PEG 1540	–	50g	–
$CaCl_2 \cdot 2H_2O$	50mg	150mg	735mg
KH_2PO_4	10mg	10mg	–
Glycine	–	–	375mg
H_2O	100ml	100ml	100ml
pH	5.7(KOH)	5.7(KOH)	10.5(NaOH)

These solutions were from Kao (1981).

and resuspended in this solution to adjust their density to 1.0×10^5 - 2.0×10^5 cells/ml. Equal volumes of each sespension were mixed in a tube. 160 μl of protoplast mixture was put on the flat bottom of 60 mm Petri dish and allowed to settle for 10 minutes. When the protoplasts had settled to the bottom of the Petri dish, 240 μl of the PEG solution (Table 2) was carefully added to the periphery of the protoplast layer, and the preparations were incubated at room temperature for 15 minutes. The PEG solution was gradually diluted by adding 160 μl, 160 μl, 320 μl and 400 μl of solution W2 or culture medium (P-5 medium) at 5 minute intervals. After another 10 minutes, the protoplasts were washed three times with culture medium. Finally 1 ml of the culture medium was added to the dishes and the dishes were sealed with Parafilm and incubated in the dark at 27℃.

RESULTS

Culture of potato tuber-derived protoplasts. The first cell divisions occurred after 3-5 days of culture, but divisions were mainly confined to densely cytoplasmic cells (Fig. 1). Sustained cell divisions were induced by addition of fresh medium (Figs. 2 and 3). When the initial cultures of protoplasts were illuminated at the intensity of 500 lux or 1,000 lux, cells did not divide at all. Some of them were elongated but finally assumed a brown color. At 3 weeks of culture, colonies reached the size of about 1 mm. When these colonies were transferred to the p-callus medium under the fluorescent light, they turned green (Fig.4). In order to induce the shoots, calli were subjected to media containing various combinations of auxin (IAA, NAA, 2,4-D) and cytokinin (kinetin, BAP, zeatin) concentrations. No shoots were formed from calli on these media as yet, but some calli showed initial shoot-like structures on the medium including 0.1 mg/1 IAA and 1 mg/1 zeatin, in the case of cv. Superior (Fig. 5). Approximately 3% and 1% of the initial plating protoplasts of cv. Superior and cv. Dejima were proliferated into the calli, respectively.

Isolation and culture of protoplasts from tobacco leaf. Several combinations of Onozuka cellulase and Macerozyme R-10 were tested to determine the optimal concentration for protoplast isolation. The yield of protoplasts was the highest when tissues were incubated in the enzyme mixture of 2% Onozuka cellulase and 1% Macerozyme R-10 (Table 3). Using a Nikon

Table 3. Effect of enzyme concentration containing 0.7M mannitol and MS. salts on protoplast isolation. Incubations were performed at 27°C for 6 hours

Conc. of enzymes		Amount of protoplasts isolated from 250 mg of leaf tissue
Onozuka cellulase	Macerozyme R-10	
1.0%	0.5%	1.9×10^6
1.5%	0.5%	2.0×10^6
1.5%	1.0%	2.8×10^6
2.0%	0.5%	2.1×10^6
2.0%	1.0%	3.2×10^6

inverted microscope, the formation of protoplasts was observed at 1 hour interval through 9 hours. The maximum protoplast yield was obtained when incubated for 6 hours in the enzyme solution and the number of protoplasts decreased thereafter. Therefore, 6 hours of incubation in the enzyme solution was thought to be optimal for protoplast isolation. The protoplasts isolated from leaf tissue are shown in Fig. 6. Exclusion staining with Evans Blue indicated that over 85% of the protoplasts were viable. Increase in cell volume as well as change in the arrangement of chloroplasts was seen on the 4th day of culture and cell division occurred after 5 days of culture (Fig. 7). When protoplasts were cultured in the dark, cell division did not occur. Chloroplasts became indistinguishable in appearance in proportion to furthur division of

cells (Fig. 8). Approximately 60% of the initial plating protoplasts were proliferated into calli. When dishes were placed under 5,000 lux rather then 1,500 lux after culture in shoot-forming media for 7 days, growth of colonies was more stimulated and they turned green. Dark green spots appeared on calli cultured for 10 days in medium Ⅲ and eventually differentiated into shoots. Shoot initiation from callus was also observed in medium I and medium Ⅱ after 3 weeks of culture. Initiation and elongation of shoots were very lagging in media I and Ⅱ when compared with medium Ⅲ. It was noticeable that callus cultured in medium I assumed a pale green color. Shoots grew rapidly in medium Ⅲ. Well developed shoots, approximately 3 cm in length, were transferred to the root-induction medium (Table 1) in order to promote root initiation (Fig.9). Plantlets which had rooted were transplanted into soil in pots and grown in a plant growth chamber. Whole plants were obtained, and they flowered (Fig. 10) and finally set seeds.

Formation of heterokaryocytes. Agglutination occurred between protoplasts after PEG treatment and the protoplasts appeared distorted (Fig. 11). It was observed that most aggregates adhered firmly to the bottom of the Petri dish, but some floated. After the dilution of PEG, most potato protoplasts returned to their normal shape, while about 70% of tobacco protoplasts were damaged. Heterokaryocytes were relatively healthy. To identify the heteroplasmic fusion products, green chloroplasts from the tobacco mesophyll protoplasts and colorless plastids and cytoplasmic strands across the central vacuole from the potato tuber cultured-cell protoplasts were used as markers. The bulk of the heterokaryocytes was relatively larger than parental tobacco or potato protoplasts (Fig. 12A, B and C). The fusion frequency of heterokaryocyte formation was about 1%. Some combinations of hormones (1 mg/1 2,4-D+0.5 mg/1 NAA+0.5 mg/1 zeation; 3 mg/1 NAA+1 mg/1 BAP; 2 mg/1 2,4-D+0.5 mg/1 zeatin) in basal P-5 medium were used to culture the heterokaryocytes. It was observed that the chloroplasts from tobacco protoplasts were dispersed into the whole cytoplasm of heterokaryocytes during culture (Fig. 13A and B) and some heterokaryocytes were elongated longitudinally (Fig. 14). The first division of heterokaryocytes took place within 5-days of culture in medium containing 2 mg/1 2,4-D and 0.5 mg/1 zeatin (Fig. 15A and B). In this medium, however, division of tobacco protoplasts was not observed.

DISCUSSION

Culture of potato tuber-derived protoplasts has not been reported as yet. Thus, the culture methods of protoplasts isolated from leaf mesophyll cells (Shepard and Totten, 1977; Binding *et al.*, 1978; Kikuta *et al.*, 1983) and from shoot-cultured cells (Thomas, 1981) were modified and applied in this study. In preliminary experiments using the agar-plating method of Nagata and Takebe (1971) with medium B of Shepard and Totten (1977), the first division of some protoplasts occurred but further division was impossible. Several combinations of hormones (NAA+ BAP; NAA+kinetin; 2,4-D+BAP; 2,4-D+kinetin; 2,4-D+zeatin; NAA+zeatin;

IAA+zeatin) were also applied, but there was no efffect. When potato tuber protoplasts were cultured, by the method of Ngata and Takebe(1971), they were enlarged in cell volume, but finally assumed a brown color and their growth became inhibited. At 15 mM sucrose of medium B, the browning of cells was intensive. At 30 mM and 60 mM sucrose, cells were more and less satisfactory but ejected cytoplasm after first cell division. Substitution of agarose for agar (Shillito *et al.*, 1983) also had no effect. Therefore, the agar-plating method of Nagata and Takebe (1971) was not suitable for culture of protoplasts from potato tuber cultured cells. Successful culture of potato tuber-derived protoplasts was possible in modified method of Thomas (1981). Frequent addition of fresh medium to the cultures stimulated the continuous division of cells to form calli. Several media containing various combianations of hormones were tested to induce shoots from callus, but failed and only rudimentary shoot-like structures occurred (Fig. 5). Studies on the induction of shoot from callus are in progress.

Growth and development of tobacco protoplasts were satisfactory in protoplast culture medium (Table 1). Callus cultured in medium I assumed a pale green color. It is thought that this was probably due to the high concentration of sucrose as compared with that of medium Ⅱ and medium Ⅲ. By comparison of medium Ⅱ with medium Ⅲ (Table 1), mannitol and IAA did not give significant effect on the induction of shoot from callus. Hayashi and Nakajima (1984) reported that they succeeded in shortening the shoot regeneration period to 4-5 weeks from tobacco protoplasts by using the method of three step cultures; cold treatment of protoplast cultures, transfer of agar blocks containing calli to the second agar medium and culture of calli in the liquid medium. Their method provided quicker results than that of Raveh and Galun (1975) and Evans *et al.* (1983) in which they could shorten the required time to 7 weeks and 6-7 weeks, respectively. In this research, visible shoot bud formation from protoplast-derived callus occurred after 35 days of protoplast culture, Thus, it seems that, in the present study, the shoot regeneration from protoplasts was relatively rapid with simple procedure.

The heteroplasmic fusion products from tobacco and potato were easily distinguishable under the Nikon inverted microscopic observation, since they shared the chloroplasts from tobacco and colorless cytoplasm and plastids from potato (Fig. 12A, B, and C). When the PEG was diluted with W2 solution, more heteroplasmic fusion products were formed than did that with P-5 medium. However, many more tobacco protoplasts were damaged by W2 solution treatment than P-5 medium treatment, while potato protoplasts were tolerant. This result was similar to that of Kao (1981) in which protoplasts from cultured cells of soybean could tolerate the enzymes, PEG, high pH and Ca^{2+} treatment very well, while the protoplasts from young leaves of *Nicotiana langsdorffii, N. glauca* and pea were much more sensitive to those conditions. This indicates that one of the reasons of the low ffrequency of heteroplasmic fusion between tobacco and potato protoplasts may be due to the serious damage of tobaccco protoplasts during fusion experiment. Preincubation of the mesophyll cells of tobaco leaves for 2-3 days in a nutrient solution or reduction of enzyme concentration used would increase the resistance of

protoplasts to PEG and high pH and Ca^{2+} solution treatment leading to higher fusion frequency (Kao, 1981). The results of this research indicate that subsequent division of heterokaryocytes would be possible if many more heterokaryocytes were obtained and cultured in more controlled conditions, though cell division of heterokaryocytes occurred once in this study.

摘 要

본 연구에서는 감자(*Solanum tuberosum* L.) 괴경의 배양세포와 담배(*Nicotiana tabacum* L.)의 엽육조직으로부터 원형질체를 분리하여 각각의 재생 여부를 확인하고 이들의 속간 원형질체 융합을 시도하였다. 그 결과 담배의 원형질체는 완전한 식물체로 재생되었고, 아직 배양해 본 예가 없었던 감자 괴경의 원형질체는 callus 상태까지 유도되었는데, 그 중 일부에서는 발생초기의 shoot로 보이는 구조가 관찰되었다.

또한 감자와 담배의 속간 원형질체 융합을 시도하여 이형 세포의 융합체를 얻었으며 그들 중 일부는 배양 5일 후에 제1차 분열을 하였음을 관찰하였다.

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(1987. 9.25 接受)

Explanation of Figures

Fig. 1. Thc first cell division of protoplast. Microsscopic examination revealed that divisions werc mainly confineed to densely cytoplasmic protoplast cellss.

Fig. 2. Multicellular colony of protoplast origin.

Fig. 3. Small callu derived from a single protoplast.

Fig. 4. Calli of protoplast origin.

Fig. 5. A rudimentary shoot like structure regenerated from callus of protoplast origin. Scalc bar equals 50 μm.

Fig. 6. Tobacco protoplasts isolatcd from leaf cells which–were incubated in the cnzyme solution of 2% Onozuka cellulase, 1% Macerozyme R 10, 0.7M mannitol and M. S salts for 6 hours.

Fig. 7. The first cell division of protoplast.

Fig. 8. Callus of protoplast origin.

Fig. 9. Plantlet of tobacco regenerated from single mesophyll protoplast.

Fig. 10. Flowers of protoplast derived plant.

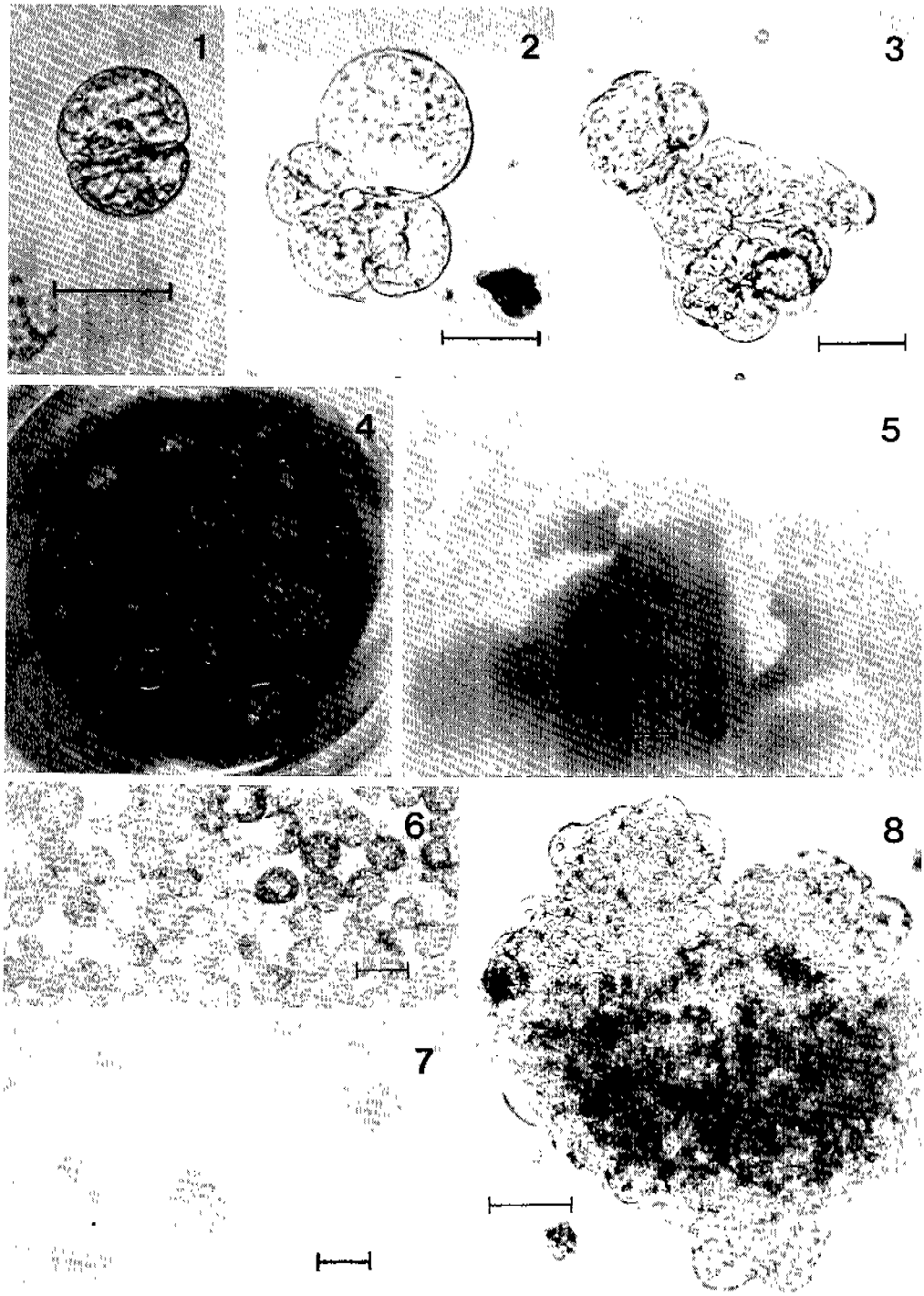
Fig. 11. Aggreation of protoplasts after PEG treatment.

Fig. 12a-c. Some fusion products (hetorokaryon) after dilution of PEG. They shared thc chloroplasts from tobacco protoplast and dense cytoplasm from potato protoplast.

Fig. 13a-b. Fusion products (hcterokaryon) which turned oval in shape during culture. The chloroplasts of tobacco protoplast origin were dispersed into the whole cell.

Fig. 14. Elongation of a fusion product (heterokayon) during culturc.

Fig. 15a-b. The first cell division of the heterokaryons after 5 days of culture in p-5 medium. Scale bar equals 50 μm.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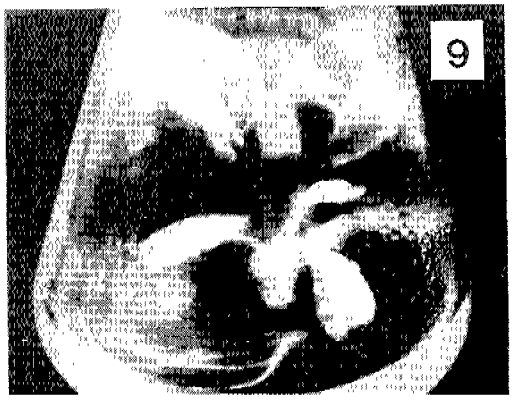
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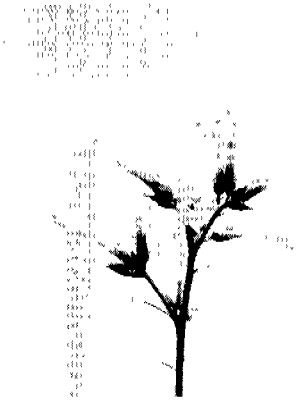
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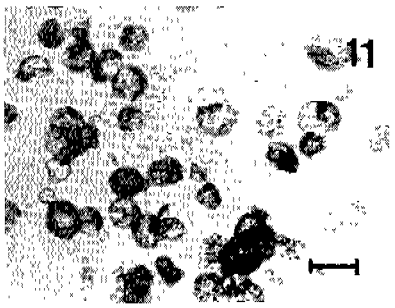
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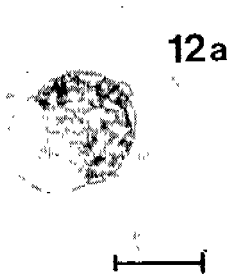
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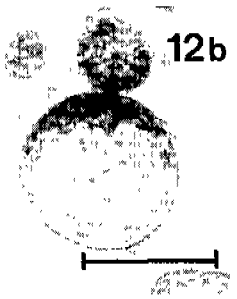
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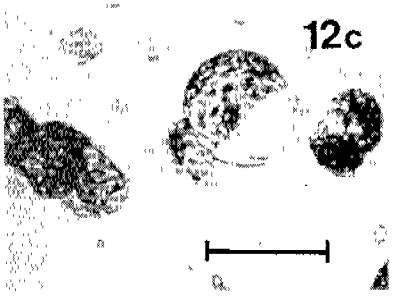
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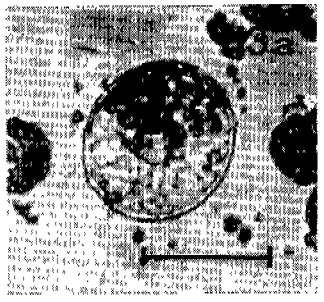
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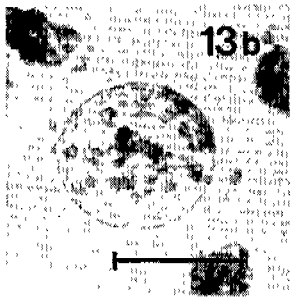
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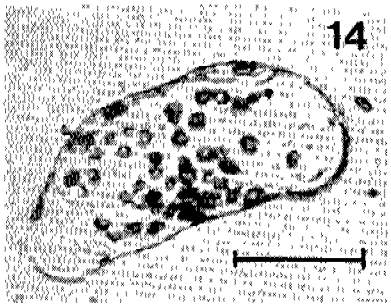
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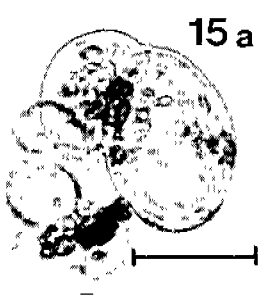
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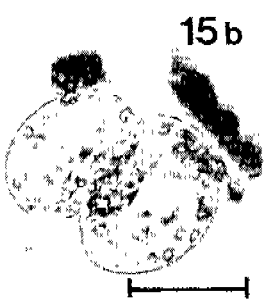
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