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Intraspecific hybridization between *Coprinus cinereus* and *Schizophyllum commune* by PEG-induced protoplast fusion and electro-fusion

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Two irreversible inhibitors, iodoacetamide and diethylpyrocarbonate, were used to select intraspecific fusion products of two mushroom species, *Coprinus cinereus* and *Schizophyllum commune*. Iodoacetamide was the more suitable inhibitor because it gave a low breakage frequency and low survival rate of the cells in the inactivation experiments. Fusion-induced by polyethylene glycol and electro-fusion were compared and, under optimal conditions, gave fusion frequencies of 16.7% to 50.0% and 6.9% to 8.4%, respectively. All fusion progeny were heterokaryons (dikaryons) and had clamp connections. There were no differences in the morphology and fruiting ability of the fusion progeny and those of the heterokaryons generated from mating.

Key words: *Coprinus cinereus*, electro-fusion, fungi, polyethylene glycol, protoplast, *Schizophyllum commune*.

Induced protoplast fusion can overcome vegetative incompatibility and produce hybrids with the combined properties of both parents. Until now, two fusion methods, polyethylene glycol-(PEG)-induced fusion and electro-fusion, have been the most widely used (Peberdy 1991). PEG can fuse a wide variety of cells, including interspecific and inter-kingdom cell types. Compared with the use of other fusogens, the PEG method is easy, inexpensive, highly reproducible and highly effective (Saunders & George 1987). In recent years, electro-fusion has been developed to increase the incidence of cell fusion (Zimmermann & Scheurich 1981; Zimmeremann & Vienken 1982). A unique aspect of electro-fusion is that it produces a high percentage of fused cells without the use of potentially cytotoxic, chemical fusogens such as PEG. In electro-fusion, cell adhesion and the induction of cell fusion can also be controlled separately. Thus, specific cell–cell interactions can be generated prior to fusion, leading to the formation of specific fusion products (Bates *et al.* 1987). Although Bates *et al.* (1987), using plant cells, suggested that the fusion frequency achieved using electro-fusion was at least an order of magnitude better than that obtained with PEG, they did not use both methods simultaneously. When San *et al.* (1990) fused mesophyl protoplasts of two genotypes of cultivated tomato using either electro- or PEG-induced fusion. They achieved fusion frequencies of 9.2% and 3.8%, respectively. Both values are in the same order of magnitude, even though the fusogen used did not involve Ca^{2+}, which facilitates the production of hybrids. Weber *et al.* (1981), however, found that the intraspecific fusion of yeast produced using electro-fusion was 10- to 100-fold better than that produced by the PEG method. Sonnenberg & Wessels (1987), investigating intraspecific electro-fusion in *Schizophyllum commune*, showed a fusion frequency of 7.8%, which was similar to the 4% achieved by Kiguchi & Yanagi (1985) with PEG-induced fusion in *Coprinus cinereus*. There have been no published comparisons of the use of the two methods with higher fungi, the subject of the present study. The present results may facilitate the application of protoplast fusion techniques in the mushroom biology and breeding.

An important step in protoplast fusion is the selection of fusion products in the regenerated colonies. The most

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widely used selection strategy is based on the nutritional complementation of both auxotrophic parents. Introduction of auxotrophic mutants or resistant mutants, however, is generally undesirable for breeding because u.v. or chemical mutagens always cause some non-specific mutation (Peberdy 1989). Although Wright (1978) used biochemical inhibitors to inactivate animal cells and produce fusion products, this method, only appears to have been used once with fungi (Sunagawa & Miura 1992). As it is unclear whether this technique leads to a 100% killing frequency (Peberdy 1989), two irreversible biochemical inhibitors were tested for selecting fusants in the present study.

Materials and Methods

Organisms

Four auxotrophic strains were used: *Coprinus cinereus* Cc5104 (his$^-$, mating type A_2B_2); *C. cinereus* Cc5026 (ade$^-$, mating type A_7B_7); *Schizophyllum commune* Sc3 (arg$^-$, mating type $A_{30}B_{30}$); and *S. commune* Sc4 (leu$^-$, mating type $A_{31}B_{31}$). The media used were: MYG [4.0 g glucose, 4.0 g yeast extract (Biolife), 10.0 g malt extract (Bacto), 14.0 g Biolife-agar, and distilled water to 1 l]; MYGM (MYG medium with 0.6 M mannitol); MM (20.0 g glucose, 2.0 g asparagin, 0.12 g thiamine-HCl, 1.0 g K_2HPO_4, 0.46 g KH_2PO_4, 0.5 g $MgSO_4$, 14.0 g Biolife-agar, and distilled water to 1 l); and MMM (MM medium with 0.6 M mannitol).

Fusogens

Three fusogens were used for protoplast fusion: Fusogen I [30% (v/v) PEG 3350 (Sigma), 0.05 M $CaCl_2$ and 0.05 M glycine, pH 8.0]; Fusogen II [30% (v/v) PEG 6000 (Sigma), 0.05 M $CaCl_2$ and 0.05 M glycine, pH 8.0]; and Fusogen III [30% (v/v) PEG 3350, 0.05 M calcium acetate and 0.05 M glycine, pH 8.0].

Inactivation Chemicals

Stock solutions of 200 mM iodoacetamide (IA) in 0.6 M mannitol and 350 mM diethylpyrocarbonate (DP) in ethanol were prepared and stored at 4°C. They were diluted in 0.6 M mannitol before use.

Inactivation of Protoplasts

Protoplast isolation and regeneration protocols were as described previously (Zhao & Chang 1993). Purified protoplasts were collected by centrifugation and mixed with 1.5 ml inactivating agent. After 5.0 min, the suspension was diluted with 4 ml 0.6 M mannitol and centrifuged for 5 min at 1000 × *g*. The pelleted protoplasts were washed again, suspended in 0.6 M mannitol, and plated (0.1 ml/plate) on MYGM to evaluate the inactivation.

PEG-induced Protoplast Fusion

About 2.0×10^7 protoplasts from each parent were combined in a fusion tube, centrifuged (1000 × *g*, 5 min) and then mixed with 0.5 ml fusogen by gentle rotation. A further 0.5 ml of fusogen was then added, and the fusion mixture mixed again by rolling. Following incubation at 30°C for 10 min, the suspension was gently diluted with 4 ml 0.6 M mannitol. Fused protoplasts were selected by centrifugation and suspended in 5 ml 0.6 M mannitol. The concentration of protoplasts and protoplast-fusion yield [PFY; the percentage of all protoplasts that were fused (Boss 1987)] were determined using a haemacytometer. Each parental strain was also fused with itself as controls. Fused protoplasts were plated on MMM and MYGM to determine fusion frequency (the number of regenerated colonies developing on MMM as a percentage of the number regenerating on MYGM).

Electro-fusion

Electro-fusion was performed in a commercial cell fusion system (DRII type; Haihua, Beijing, China). The fusion chamber consisted of a microscope slide with two, parallel, gold-plated electrodes, either 100 or 200 μm apart, connected to a power supply. For fusion, 30 μl protoplast suspension (10^8 protoplasts/ml) was allowed to settle to the bottom of the fusion chamber for 5 min. Protoplasts were aligned in chains by dielectrophoresis in an alternating-current (a.c.) field with a frequency of 2 MHz and a field strength of 50 to 80 V/cm. After preliminary experiments to optimize the conditions, fusion was initiated by the application of one or two direct-current (d.c.), square-waved pulses, each of 20 μs and with a field strength of 8.0 kV/cm. After the pulses, the a.c. field was gradually decreased to 0 V over a period of 10 s. Protoplasts were left in the fusion chamber for 2 or 4 min until rounding of the fused protoplasts was complete. The concentration of protoplasts, PFY and fusion frequency were determined as for PEG-induced fusion.

Comparison of Mycelium Growth Rate

Ten heterokaryotic produced by each method were chosen at random and three, 2-mm diameter block were taken from each inoculate plate and transferred onto three freshly prepared plates. Colony diameters were measured after 3 days. To observe the effect of mitochondria, an inoculate from Cc5104 and one of Cc5026 were placed on a new plate at a distance of 2 cm from each other. After 3 days, three plugs of mycelia were taken: one from the Cc5104 side, one from the Cc5026 side and one from the contact zone. These blocks were transferred into new plates and cultured at 30°C for another 3 days. Colony diameters and fruiting ability (see below) were then compared.

Fruiting Test

The fruiting ability of the different heterokaryons from the two fusion methods were compared in a fruiting test. The heterokaryons generated by mating were used as the controls. In the fruiting test, all cultures were transferred into MYG medium and incubated at 25°C with alternate 12-h light/3-day dark periods. Fruiting time was recorded once a fruiting body formed.

Results

Inactivation by Iodoacetamide and Diethylpyrocarbonate

Iodoacetamide (IA) and diethylpyrocarbonate (DP) were screened for their abilities to introduce complementary lesions which could be rescued by the cell fusion. The compatible monokaryotic strains, Cc5104 and Cc5026, were used to observe their effect. The optimum concentration of IA was 20 to 50 mM, which gave a survival frequency of $< 10^{-5}$ and a breakage of protoplasts of < 30% (Figures 1 and 2). Although the effective DP concentration was between 10 and 35 mM, the percentage breakage for treated protoplasts was > 80% in this range (Figures 3 and 4). Accordingly, 50 mM IA and 7 mM DP were used in subsequent fusion experiments.

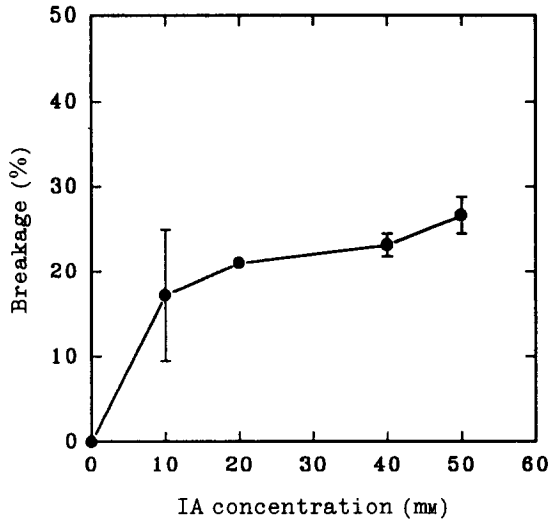

Figure 1. Protoplast breakage of Cc5104 after the addition of iodoacetamide (IA).

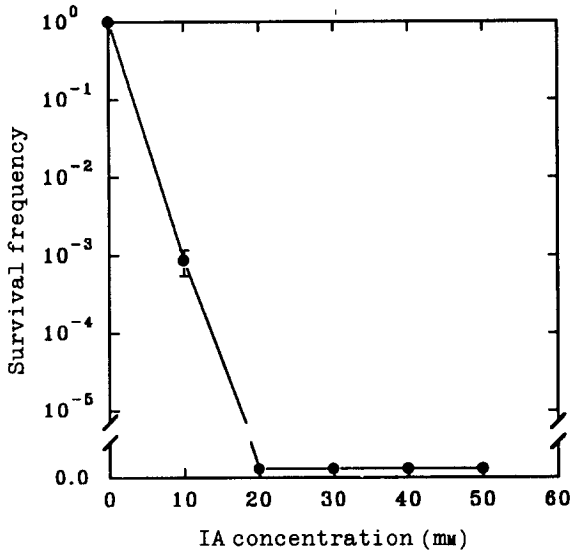

Figure 2. The survival frequency of Cc5104 protoplasts after inactivation by iodoacetamide (IA).

Analysis of Progeny

Fusion was carried out between compatible pairs so that fusion progeny could be easily identified by growth in the minimal medium. About 1000 progeny from each fusion were checked for the occurrence of clamp connections and all were found to be dikaryons. No monokaryotic heterotrophs were detected. In self-fusion experiments, no heterotrophs were found in MMM.

Effect of Different Fusogens on Fusion Frequency (Table 1)

Calcium acetate can increase fusion frequency in yeast (Kavanagh *et al.* 1991) and was therefore also used in this study. If no fusogen was added, no heterokaryons were obtained. In the presence of PEG with calcium acetate, PFY and fusion frequency were 41.9% and 36.9%, respectively. In the presence of PEG with $CaCl_2$, PFY and fusion frequency were similar ($p > 0.05$), at 35.7% and 41.1%, respectively. As PEG 6000 was found to be less efficient than PEG 3350, only fusogen I was used in subsequent experiments.

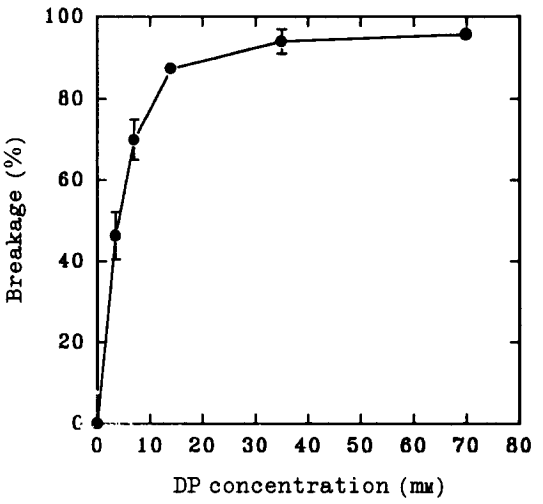

Figure 3. Protoplast breakage of Cc5026 after the addition of diethylpyrocarbonate (DP).

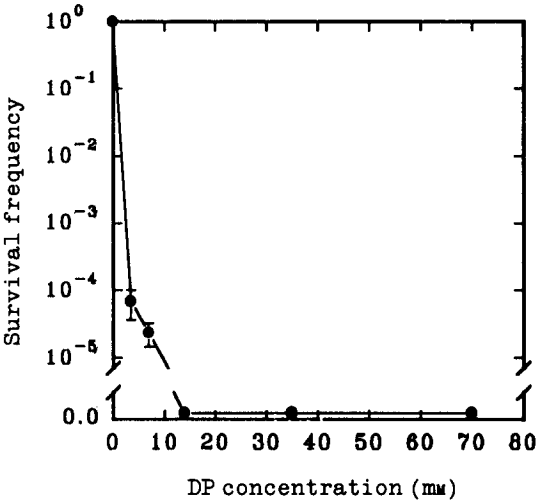

Figure 4. The survival frequency of Cc5026 protoplasts after inactivation by diethylpyrocarbonate (DP).

Effect of Different Fusion Protocols on Fusion Frequency

The more usual, single addition of PEG (Anné and Peberdy 1975) gave a PFY and fusion frequency of 18.9% and 18.1%, respectively. Step-wise addition of PEG, however, increased PFY and fusion frequency to 29.4% and 28.1%, respectively.

Optimization of Electro-fusion

All electro-fusion processes could be observed under the microscope and the optimization of electrical parameters

Table 1. Effect of different fusogens on the frequency of PEG-induced protoplast fusions in *C. cinereus*.

Fusogen*	Protoplast fusion yield (%)	Fusion frequency† (%)
Control (no PEG addition)	3.8	0.0 ± 0.0
Fusogen I	35.7	41.1 ± 2.5
Fusogen II	27.3	29.5 ± 7.6
Fusogen III	41.9	36.9 ± 2.1

* See Materials and Methods.
† Values are means from triplicated experiments ± standard deviation.

Table 2. Effect of the pulse height of the d.c. field on fusion frequency.

Pulse height (kV/cm)	Fusion frequency* (%)
0.0	0.0 ± 0.0
4.0	4.7 ± 1.7
8.0	6.9 ± 1.9
12.0	4.9 ± 2.1

*Values are means from triplicate experiments ± standard deviation.

was easily attained. Microscopical observation indicated that use of the chamber with a smaller electrode distance (100 μm) led to more protoplast chains and better membrane contacts. Dielectrophoresis was carried out by slowly increasing the a.c. field (2 MHz) to 80 to 120 V/cm. Further increases of the field strength caused rotation. When the electrical parameters of the d.c. field, such as field strength and the length, number and intervals between the pulses, were optimized microscopically, field strength was found to be the critical parameter (Table 2); the optimal strength was 8 kV/cm.

Fusion Frequency achieved by PEG and Electro-fusion (Table 3)
In PEG-induced fusion experiments with *C. cinereus*, no heterokaryons occurred in regenerated progeny if no fusogens were not added but fusion frequencies of > 35% were achieved when fusogens were present. This compares with a value of only 7.0% when electro-fusion was used. For *S. commune*, the fusion frequencies from PEG-induced fusion and electro-fusion were 16.7% and 8.4%, respectively. Inactivation of the protoplasts did not affect fusion frequency, except for the fusion between Cc5104 and Cc5026 when both strains were inactivated.

Comparison of Colony Diameters and Fruiting Ability
To distinguish the different heterokaryons resulting from PEG-induced fusion and electro-fusion, their colony sizes and fruiting time were scored (Table 4). Three types of heterokaryons from hyphal anastomosis were also chosen for the investigation of the effect of mitochondria. The heterokaryon from the Cc5104 side had the fastest fruiting time, even though its growth speed was not the fastest. Fusion method had no significant effect on colony size or fruiting time.

Discussion

PEG appears to be a universal promoter of membrane fusion, effecting animal cells, and the protoplasts of plants, fungi and bacteria (Saunders & George 1987). It is a polymeric compound with a range of molecular weights. The popular use of PEG for fusions was due to its dual role

Table 3. Comparison of fusion frequency in PEG-induced fusion and electro-fusion.

Fusion method and strain(s) used	Protoplast fusion yield (%)	Fusion frequency* [...]
Control (no PEG addition):		
Cc5026 + Cc5104	3.8	0.0 ± 0.0
PEG-induced fusion:		
Cc5026 + Cc5104	34.3	39.3 ± 2.5
Cc5026 + Cc5104 (inactivated by IA)	35.3	50.0 ± 6.9
Cc5026 (inactivated by IA) + Cc5104	25.0	42.3 ± 3.8
Cc5026 (inactivated by IA) + Cc5026 (inactivated by DP)	NT	0.0 ± 0.0
Electro-fusion:		
Cc5026 + Cc5104	6.6	7.2 ± 2.4
Control (no PEG addition):		
Sc3 + Sc4	NT	0.0 ± 0.0
PEG fusion: Sc3 + Sc4	23.8	16.7 ± 8.7
Electro-fusion: Sc3 + Sc4	6.7	8.4 ± 2.5

*Values are means from triplicate experiments ± standard deviation.
NT—Not tested.

Table 4. Comparison of colony diameters and fruiting times in different dikaryons of *C. cinereus*.*

Dikaryon type	Colony size (mm)	Fruiting time (days)
Cc5026 × Cc5104		
Contact zone	58 ± 1	15 ± 1
Cc5104 side	59 ± 1	15 ± 1
Cc5026 side	59 ± 1	11 ± 1†
PEG fusion		
Cc5025 + Cc5104	60 ± 1	18 ± 4
Cc5104 + Cc5026 (inactivated by IA)	62 ± 1	17 ± 4
Cc5104 (inactivated by DP) + Cc5026	60 ± 1	16 ± 4
Electro-fusion		
Cc5026 + Cc5104	60 ± 2	16 ± 4

*Each value is the mean of 10 results ± standard deviation.
†Value significantly different from that for contact zone ($p < 0.05$).

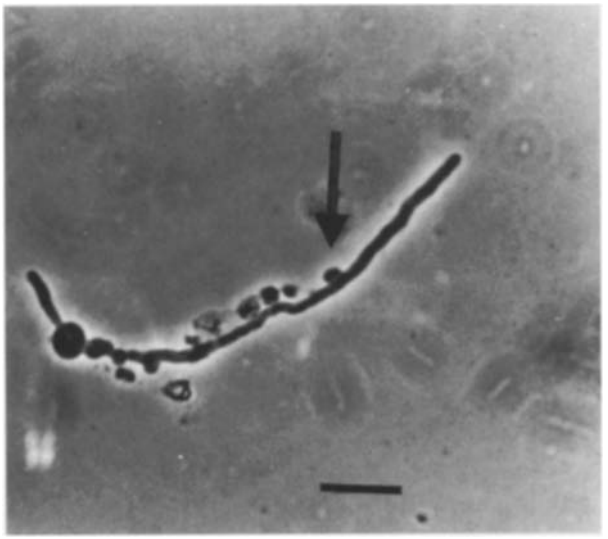

Figure 5. The appearance of a clamp connection (↓) in a regenerated protoplast after PEG-induced fusion between strains Cc5104 and Cc5026. Bar = 10 μm.

both as a cell agglutinator and a membrane modifier. The most commonly used fusion procedure in fungi involves the use of PEG, high calcium concentrations and high pH values (Peberdy 1989). In the present study, PEG-induced fusion frequencies were > 16.7% whereas those achieved by electro-fusion, were only 6.9% to 8.4%, even under optimized condition, similar to those recorded by Sonnenberg & Wessels (1987) for *S. commune* after electro-fusion. Why was the PEG-induced fusion frequency higher? Perhaps because the fusion progeny came from post-fusion anastomosis instead of the protoplast fusion; hyphal anastomosis can give rise to heterokaryons. In the electro-fusion experiment, it was clear that post-fusion anastomosis did not occur because treated protoplasts did not aggregate. After PEG-induced fusion, however, fused protoplasts clumped together and could not be separated during the regeneration. To ensure that the observed heterokaryons did come from fused protoplasts and not from post-fusion anastomosis, fused protoplasts produced by PEG-induced fusion were observed microscopically and scored for the occurrence of clamp connections in freshly regenerating protoplasts (Figure 5). The real fusion frequency (the percentage of regenerating protoplasts that had clamp connections) was then determined as 20% to 30%, consistent with the earlier results (Table 5). When the inactivated protoplasts were fused with an auxotrophic partner, only the fused protoplasts could regenerate in minimal media. It remains unclear why electro-fusion did not give better results than the PEG-induced fusion. It seems that, although electrofusion has valuable attributes, the form used here was sub-optimal. The electrical fields used to induce fusion may cause irreversible membrane damage if they are not carefully controlled. Secondly, the volume of the electrofusion chamber used in the present study was very small and restricted the number of fused products. Thirdly, many protoplasts may have been hidden in the corners of the chamber and been unavailable for fusion.

Mitochondrial inheritance has been observed in *C. cinereus* and other basidiomycetes by May & Taylor (1988) and Smith *et al.* (1990). They found that mitochondria did not migrate along with nuclei during mating and they failed to observe intracellular mixed or recombinant mtDNA molecules. The effect of mitochondria on fruiting ability remains unknown, however. The present, preliminary results indicate that mitochondrial inheritance may affect the formation of fruiting bodies.

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