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Heterokaryon formation in the basidiomycete *Schizophyllum commune* by electrofusion of protoplasts

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Summary. Conditions for high frequency electrofusion of protoplasts from the basidiomycete *Schizophyllum commune* are described. Visual inspection revealed up to 30% of the protoplasts engaged in fusion. Using complementing nutritional mutations, nearly 7% of the regenerated protoplasts could be recovered as heterokaryotic mycelia. The method is probably equally applicable to other basidiomycetes such as *Agaricus bisporus*, permitting the recovery of fusion products in the absence of selection markers.

Key words: Electrical fusion – Protoplasts – Heterokaryon formation – *Schizophyllum commune*

Introduction

Fusion of fungal protoplasts is now a widely used technique to produce heterokaryons. The technique can be used to overcome vegetative incompatibility (Dales and Croft 1977) and to produce hybrids with combined properties of the parents (Anné 1982). In most cases, fusion of protoplasts of filamentous fungi is induced by polyethylene glycol (PEG) (Peberdy 1980; Ferenczy 1984) and hybridization experiments are usually carried out using complementation of different auxotrophic mutations. The reason for this is the relative low fusion frequency and 'clustering' of protoplasts. The need for mutants is a disadvantage because mutagenesis is often accompanied by the loss of desirable properties. Besides, in commercially important strains, like the white button mushroom *Agaricus bisporus*, it is difficult to obtain and genetically analyse mutants because of multinucleated cells and the scarcity of homokaryotic basidiospores. Electrofusion has been developed (Zimmermann and Scheurich 1981) to increase the incidence of cell fusion. This procedure has been reported to be a rapid and efficient method to fuse cells of mammalian origin (Lo et al. 1984; Vienken and Zimmermann 1985) and protoplasts of plants (Zimmermann and Scheurich 1981; Bates et al. 1983; Tempelaar and Jones 1985; Puite et al. 1985; Watts et al. 1985) and yeasts (Schnettler and Zimmermann 1985; Förster and Emeis 1986). The high fusion percentage permits selection by micromanipulation of differentially labelled protoplasts or by screening isoenzyme banding patterns of random fusion samples, as suggested by May and Royse (1982).

To test the suitability of this technique for the fusion of protoplasts from basidiomycetes, we used protoplasts of *Schizophyllum commune* as a first approach because of the availability of non-leaky auxotrophic mutants.

Materials and methods

Strain and culture conditions

Compatible auxotrophic strains of *Schizophyllum commune* (A2B1 ura-1 and A4B47 nic-3) were maintained on minimal agar medium (MM, Dons et al. 1979) supplemented with 0.3% yeast extract (Difco). Cultures for inoculation were grown in Petri dishes on minimal agar medium supplemented with 1 mM uracil or 1 mM nicotinamide, as required, at 24 °C for 10 days. The contents of one Petri dish were homogenized in 50 ml supplemented liquid minimal medium (Waring blendor, 2×30 s, full speed). The homogenate was added to 200 ml supplemented minimal medium in a 1 l Erlenmeyer flask and incubated for 22 h at 24 °C on a rotary shaker (250 rpm).

Wall-lytic enzymes

Wall-lytic enzymes for protoplast production were isolated from the culture fluid of *Trichoderma harzianum* (CBS 35433) as described (De Vries and Wessels 1972) with the exception

that the wall substrate for growth of *T. harzianum* was derived from *Penicillium chrysogenum*. This wall substrate was prepared as follows. *P. chrysogenum* waste (kindly supplied by Gist-Brocades, The Netherlands) was stirred in 1 M NaOH at 60 °C for 1 h at a concentration of 100 g (dry wt)/l. The suspension was acidified to pH 5.0 with glacial acetic acid and filtered over glass-filter paper. The material on the filter was extensively washed with water, freeze-dried and used in the *T. harzianum* growth medium as the wall substrate at a concentration of 5.0 g/l. The isolated lytic enzyme preparation could be used at a concentration of 1 mg/ml for rapid protoplast release. It caused minimal damage to protoplasts, as evidenced by continuation of nuclear divisions in the protoplasts in the presence of these enzymes (Van der Valk and Wessels 1973) and the high regenerative capacity of the protoplasts (De Vries and Wessels 1975). A similar preparation of *T. harzianum* is commercially available (NovoZym 234) but we found a lower stability in the protoplasts released with this enzyme mixture than with the enzyme preparation prepared as described above. We did not test the commercial preparation in the experiments to be described.

Preparation of protoplasts

Each culture was harvested by filtration on nylon cloth (150 μm mesh width), rinsed with 100 ml 0.5 *M* $MgSO_4$ and suspended in 100 ml 0.5 *M* $MgSO_4$, 1 mg/ml wall-lytic enzymes and 1 m*M* $CaCl_2$. After stationary incubation for 16 h at 24 °C in Petri dishes (14.5 cm, 50 ml per dish), the suspensions were centrifuged in screwcapped tubes (15 min, 600 *g*) and the floating protoplasts were collected with a pipette. Due to the high conductivity of the protoplasting medium, protoplasts were transferred to MCT medium (0.5 *M* mannitol, 1 m*M* $CaCl_2$ containing 50 μg/ml wall-lytic enzymes) and washed several times by sedimentation (15 min, 500–600 *g*). The final pellet was suspended in 1 ml MCT and the number of protoplasts from both strains were counted in a hemocytometer. Between 0.5 and 1.0×10^6 protoplasts of each strain were mixed (1 : 1) and washed once with 14 vol MCT. Finally, the protoplasts were resuspended in 1 ml MCT.

Electrofusion

The fusion chamber consisted of a microscope slide with two parallel chromium plated steel electrodes 1.5 mm apart connected to a power supply, CFS-17, constructed at the Central Electronic Department of the University of Groningen. For fusion, 30 μl of the protoplast suspension (3 to 6×10^4 protoplasts) was pipetted into the fusion chamber and allowed to settle on the bottom for 5 min. Protoplasts were aligned in chains by dielectrophoresis in an AC-field with a frequency of 2 MHz and a field strength of 100–130 V/cm. Fusion was initiated by application of three DC square waved pulses, duration 20 μs, with intervals of approximately 0.4 s, and a field strength of 2.0 kV/cm. After application of the DC pulses the AC field was gradually decreased to zero over a period of 10 s and the protoplasts were left in the fusion chamber for 2 to 4 min until microscopic examination showed that fusion was complete (rounding up of fused protoplasts). The protoplasts were then removed with a pipette, the fusion chamber rinsed twice with 60 μl MCT and the pooled fractions diluted with molten agarose medium (MM, 0.8% low melting point agarose, 0.5 *M* $MgSO_4$, 35 °C) to a concentration of 1,000 to 2,000 protoplasts/ml. These suspensions were plated on regeneration medium (MM, 1.5% purified agar, 0.5 *M* $MgSO_4$, 1 ml per Petri dish) containing 1 m*M* CsCl. Regeneration capacity of the protoplasts after fusion was calculated by plating a 10 times diluted suspension on regeneration medium supplemented with both 1 m*M* uracil and 1 m*M* nicotinamide. To check for spontaneous fusions, protoplasts only subjected to dielectrophoresis were plated on regeneration medium.

Results and discussion

Microscopic observations

For optimal fusion, as many protoplasts as possible have to be collected in chain-like aggregates. Only protoplasts in these chains make good membrane contact: a prerequisite for a successful fusion (Zimmermann and Vienken 1982). It appeared that this was best done by allowing the protoplasts to settle on the bottom of the fusion chamber for 3 min prior to dielectrophoresis. This also made microscopic observation of the fusion process easier because most of the protoplasts were then in one plane.

Dielectrophoresis was carried out by slowly increasing the AC field (2 MHz) to 100–130 V/cm. Further increase of field strength caused circulation of the medium between the electrodes. Up to 80% of the protoplasts could thus be collected in chains which varied in length between 2 and 24 protoplasts (Fig. 1).

The electrical parameters of the DC field, such as the height, length, number and intervals between the pulses were first optimized microscopically by scoring the fusion events in the chains. This was easily done because fusion products could be recognized even after rounding up by virtue of the presence of multiple vacuoles which did not immediately fuse (Fig. 2). The electrical parameters for optimal fusion were: pulse length 20 μs, pulse number 3, pulse interval approx. 0.4 s and a pulse height of between 1.4 and 2.2 kV/cm. Furthermore, it appeared that the number of fusing protoplasts was increased by low concentrations (50 μg/ml) of wall-lytic enzymes. This was probably due to the prevention of wall regeneration which is very rapid in *S. commune* in the absence of wall-lytic enzymes (De Vries and Wessels 1975). Any wall formation prior to or during attempted fusion would inhibit fusion because of impairment of membrane contact. With the electrical parameters mentioned above and in the presence of 50 μg cell-wall lytic enzymes per ml, up to 30% of the protoplasts in the chains were visually involved in fusions.

Heterokaryon formation by electrofusion of protoplasts

The two monokaryotic strains of *S. commune* used in this study contain different alleles of the incompatibility genes and thus would normally anastomose to form a stable heterokaryon with each cell containing one of both nuclear types, i.e. a dikaryon (Raper 1983). The introduction of different nutritional mutations in the

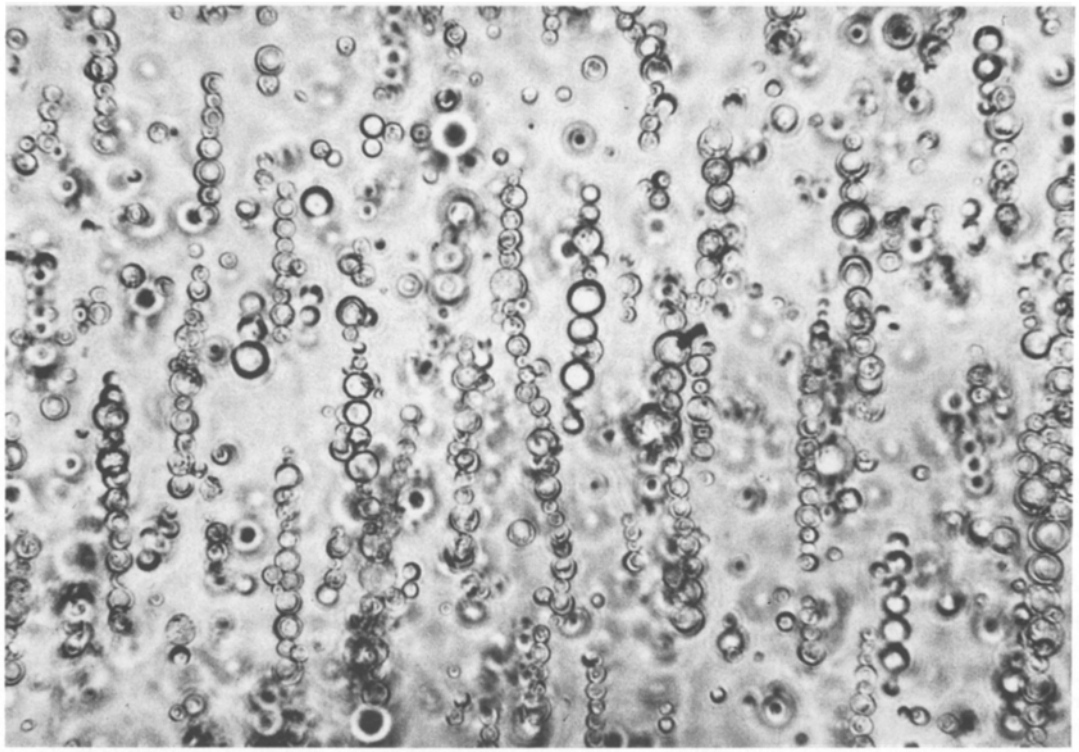

Fig. 1. Alignment of *S. commune* protoplasts by dielectrophoresis in an AC-field with a frequency of 2 MHz and a field strength of 100–130 V/cm

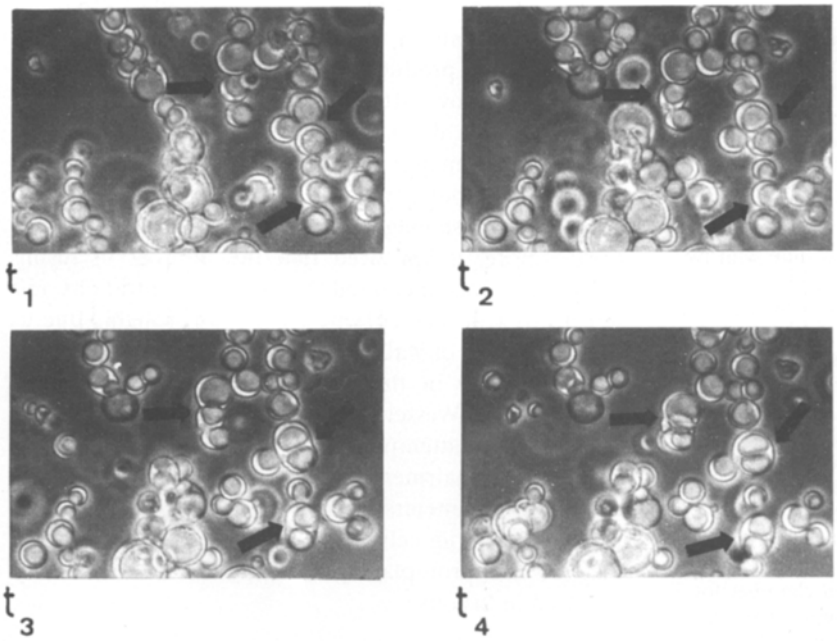

Fig. 2. Fusion sequence of protoplasts of the ura-1 and nic-3 strains of *S. commune*. Electrical parameters are described in "Materials and methods". t_1: immediately after the DC pulses; t_2–t_4: 30, 60 and 90 s later

monokaryons allowed for a convenient selection system because of the complementation of the mutations in the dikaryon resulting in growth on minimal medium. Since hyphal anastomoses could give rise to heterokaryons it was essential to ascertain that after fusion of protoplasts, regeneration started from single protoplasts. In the electrofusion experiment this criterion was met because, unlike in polyethyleneglycol-induced fusions, the protoplasts did not clump and could be plated as single units. To prevent any secondary fusions after regeneration of colonies, CsCl was included in the regeneration medium preventing extensive growth of

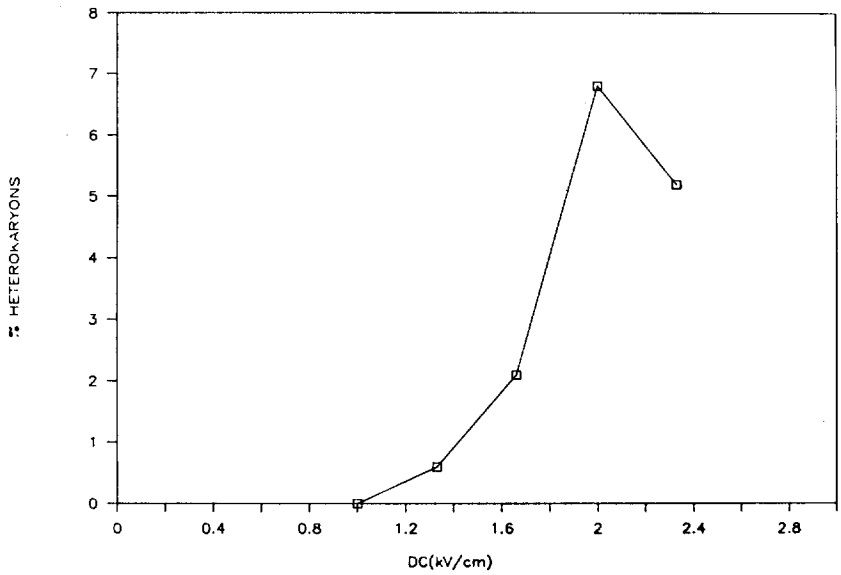

Fig. 3. Percentage complementing heterokaryons (ratio of the numbers of colonies appearing on minimal and supplemented regeneration medium) formed at varying pulse heights of the DC-field. Other electrical parameters are described in "Material and methods"

the colonies (Tilburn et al. 1983). This also made counting of the colonies easier. To determine the optimal pulse height for heterokaryon formation the fusion experiments were carried out as described above with the pulse height varying between 1.00 and 2.33 kV/cm. The frequency of heterokaryon formation was calculated as the ratio of colonies visible after 5 days on minimal and supplemented regeneration medium, respectively. As shown in Fig. 3 there was a clear optimum of 6.8% complementing heterokaryons at a DC field strength of 2.0 kV/cm. In addition, there was a threshold value for electric field-induced fusion around 1 kV/cm. Pulses with a field strength above 2 kV/cm probably caused irreversible membrane breakdown because at these high field strengths the number of lysed protoplasts increased.

Mixtures of protoplasts not submitted to the field-induced fusion, but otherwise treated similarly, appeared to regenerate a wall after plating on minimal medium but only produced slow growing hyphae which stopped after generation of a few cells. Even after prolonged incubation (2 weeks) only a few colonies appeared (less than 0.05% of those produced under fusion) and these could have arisen by fortuitous hyphal anastomoses.

If there are binary fusions and 6.8% of the regenerates are heterokaryons, then about 20% of the protoplasts must have been involved in fusion. This is somewhat fewer than observed with the microscope (up to 30%) but there was variability in this respect between experiments and there were always some protoplasts that lysed after the application of the DC pulses. In addition, microscope examination revealed many fusion events involving more than two protoplasts. This would reduce the number of heterokaryons expected from only binary fusions. This could also result in the formation of fusion products with disparate ratios of the complementing nuclei, which could affect the growth rate on minimal medium of mycelia derived from them. In fact, microscope examination of regenerating protoplasts showed that many more mycelia started to grow than eventually produced macroscopic colonies on minimal medium.

Although the heterokaryotic fusion products showing nutritional complementation all eventually produced dikaryotic mycelia in which each cell contained one of both the complementing nuclei, there was a surprisingly long delay before the dikaryotic phenotype arose. At least during the first 3 days of growth the heterokaryotic mycelia showed no sign of clamp connections and generally exhibited the homokaryotic phenotype. We observed this phenomenon earlier with heterokaryons produced by polyethyleneglycol-induced fusions (H. L. Hoeksema, C. A. Raper and J. G. H. Wessels, unpublished data). Unfortunately nothing is known about the distribution of complementing nuclei in these non-clamped heterokaryons. Apparently, although interaction of the nuclei clearly results in complementation of the nutritional deficiencies, it takes hundreds of cell generations before the interaction of the different incompatibility alleles is manifested by the formation of the dikaryon. A disturbing possibility would be that complementing nuclei in heterokaryotic protoplasts segregated into mixed homokaryotic mycelia which grow by cross-feeding and only later produce dikaryotic cells by hyphal anastomoses. Only careful microscopic analysis or analysis of isozyme patterns can resolve this. At present we favour the assumption that the delay of dikaryosis is an intrinsic property of the control of dikaryosis by the incompati-

bility genes. A similar delay in the expression of the dikaryotic morphology has been noted after germination of homokaryotic basidiospores of a strain of *S. commune* carrying mutations in both the A and B-incompatibility genes (Koltin 1970). Conversely, separation of the nuclei of a normal dikaryon by protoplast formation leads to continued expression of partial clamp formation in the homokaryotic revertants for some time, although different alleles of the incompatibility genes are no longer present (Wessels et al. 1976).

The precise course of events after fusion of protoplasts in the formation of stable heterokaryotic mycelia remains to be established. However, the high frequency of formation of heterokaryons by electrofusion of protoplasts of *S. commune* encourages the use of this method for producing heterokaryons in other basidiomycetes such as *A. bisporus,* for which no nutritional markers are available. A satisfactory protoplasting-regeneration system for this species has now been developed (to be published) and visual inspection of *A. bisporus* protoplasts under electrofusion conditions as described in this paper revealed the same high frequencies of fusions as noted for *S. commune.*

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