INTRASPECIFIC HYBRIDIZATION IN VOLVARIELLA VOLVACEA BY PROTOPLAST FUSION TECHNIQUE

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Abstract

Methods for the isolation of protoplasts from *Volvariella volvacea* using *Trichoderma harzianum* liquid culture was evaluated. Addition of chitin, laminaria meal and hyphal walls from the fungus stimulate synthesis of both chitinase and B-glucanase activities which resulted in high protoplasts yield. A comparison of protoplasts yields in different stabilizer system was described. Reversion frequency was shown to be influenced by nutrient composition especially carbon and nitrogen source in the regeneration medium. Investigation of the factors affecting fusion frequency indicate that polyethylene glycol concentration (PEG), pH and temperature were important. Proof of heterokaryosis in fused protoplasts was evident by the recovery of recombinant progenies after meiotic segregation from two nutritionally complementary auxotropbic markers.

Introduction

Studies on the mass cultivation of the edible mushroom *Volvariella volvacea* have been actively carried by workers in tropical and subtropical regions (1-3). However, interest in the genetics and breeding of this economically important fungus has not been developed despite the pioneering work of Chang and Yau (4) on its life history.

In recent years several studies have shown the possibility of obtaining successful complementation between nuclear genes, especially among fungal strains having incompatible mating factors by using the protoplast fusion technique (5). Successful crosses have been achieved with many species, including some of industrial importance, e.g. *Saccharomyses cerevisiae*, *Schyosacchromes pombe*, *penicillium species* and *Candida* tropicales (6). The significant development of fusion technology made at intraspecific level may enhance the possibility of improving different commercial mushroom strain.

A comprehensive investigation was therefore undertaken to demonstrate heterokaryon formation following induced intraspecific fusion between protoplast of nutritionally complementary auxotrophs of *V. volvacea*, using solutions containing polyethylene glycol. Additional information derived from this work may be of great value to potential mushroom investors and may lay the foundation for future breeding experiments which would undoubtedly lead to the discovery of high yielding strain.

Materials and Methods

Culture media

A range of regeneration media osmotically stabilized with either $MgSO_4$ or KCl was prepared. Tests for different carbon sources were conducted using sugar concentration of: glucose, sucrose, dextrose, D-galactose, L-xylose, lactose and mannose. Sugars were autoclaved separately prior to incorporation in mushroom minimal medium (7). Source materials for nitrogen were as follows: yeast, peptone, asparagine, urea, potassium nitrate and monobasic ammonium phosphate. The amount of different nitrogenous substances added to the agar media was based on computed nitrogen contents of the sample to give lg^{-1}. All media were sterilized for 15 min. at 15 psi and the pH value was adjusted as required.

Organism

Auxotrophic mutants of *V. volvacea* were maintained on a mushroom minimal medium supplemented with the growth requirements of the organism. *Trichoderma harzianum* was maintained routinely in TLE medium as described by Peberdy and Isaac (8).

Treatment with mutagen

Protoplast suspensions were exposed to U.V. lamp (with emission of 39 erg $sec^{-1}cm^{-2}$) with constant agitation using a magnetic stirrer, and a distance of 30 cm from the lamp. Exposure was terminated at varying time and the suspensions were suitably diluted before plating on to complete mushroom medium (7). A non-irradiated sample was used as a control.

Protoplast Isolation

Lytic enzyme from *T. harzianum* was prepared according to the procedure of Santiago (9). Mycelium for protoplast isolation was grown in submerged culture in minimal medium at 28°C. After 3 days incubation it was harvested by filtration, washed three times in buffer/stabilizer solution and dried by vacuum. It was resuspended in a digestion mixture of equal volumes of buffer -stabilizer solution and *Trichoderma* liquid enzymes, at a final concentration of 20 mg fresh weight ml^{-1}. Digestion mixtures were incubated at 28°C on a reciprocal shaker for 3-4 h.

Protoplast reversion

The frequency of protoplast reversion was determined as colony counts from diluted protoplast preparations. Comparison

was made using minimal mushroom medium containing different carbon and nitrogen sources in an attempt to optimize reversion.

Fusion Treatment and Selection of Heterokaryons

Protoplasts of two nutritionally complementary strain were mixed after washing and centrifuged for 10 min. The protoplast pellets were resuspended in 1 ml 30% (w/v) polyethylene glycol, mol. wt. 6000 (PEG) (Koch-Light), in 0.01 M $CaCl_2$ and 0.05 M glycine, adjusted to pH with 0.01 M NaOH. After incubation for 10 min. in a water bath at 30°C, the suspension was diluted with 4 ml stabilizer solution and centrifuged at 700 g for 5 min. The protoplasts were washed twice with 0.6 M $MgSO_4$ and finally resuspended in 5 ml of the same stabilizer system. Serial dilutions of treated protoplasts were plated on hypertonic minimal medium and complete medium to select heterokaryons and to count the total number of viable protoplasts, respectively.

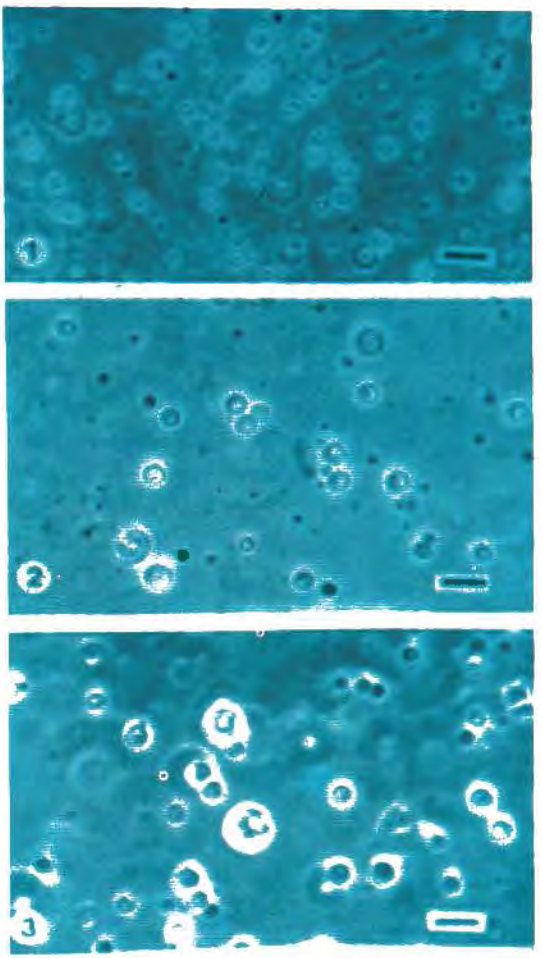
Fruit-body production

Heterokaryons produced following protoplast fusion were used in fruiting experiments. Rye gain spawn was prepared as earlier reported (9) and after sterilization was inoculated with agar plug taken the margin of a growing colony. The fruiting ability of the heterokaryons was tested by culture on wheat straw.

Results and Discussions

Variations in protoplast yield with different inducer substrate supplements used in TLE production.

Various growth substrates were incorporated in the basic TLE liquid culture and tested for effectiveness in producing protoplasts from *V. volvacea* (Fig. 1). Compared with media containing chitin and laminaria meal, the addition of *V. volvacea* hyphal walls had a significant effect on enzyme production and in turn on the protoplast yield. The maximum level of enzymatic digestion with this supplement was reached after 4 h incubation, the peak occurring in $MgSO_4$ stabilizer. With both laminaria meal and chitin supplemented separately to the hyphal walls there is an increase in protoplast production. Addition of hyphal walls to the culture medium show higher chitinase activity, but lower α and β-glucanase activity than the enzyme produced on the other substrate (Table 1). Enzymes produced using a total mixture of inducer substrates, i.e. hyphal walls, laminaria meal and chitin was active in mycelial lysis resulting in total fragmentation and disintegration of the hyphal. This preparation stimulated synthesis of both chitinase and β-glucanase activities producing high protoplast released from this fungus.



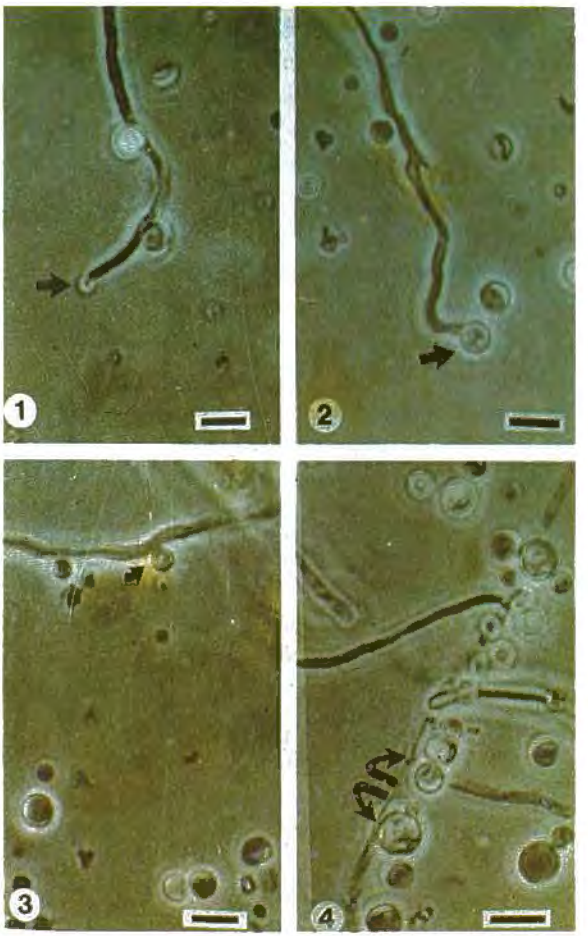
Morphology of protoplasts released during incubation of *V. volvacea* mycelium in 0.6 M $MgSO_4$ stabilized lytic mixture at 28°C.

Protoplast photographed in the lytic mixture, phase contrast microscope

(1) Protoplasts liberated after 1 hr digestion, small and dense

(2) Protoplast after 2 hr have uni- and multi-vacuolate types

(3) After 3 hr the protoplasts were large and highly vacuolate. Bar markers represent 5μ.



Morphology of protoplasts emerging and liberated from *V. volvacea* mycelium during incubation in 0.6 M. $MgSO_4$ and 0.6 M kcl stabilized lytic mixtures at 28°C.

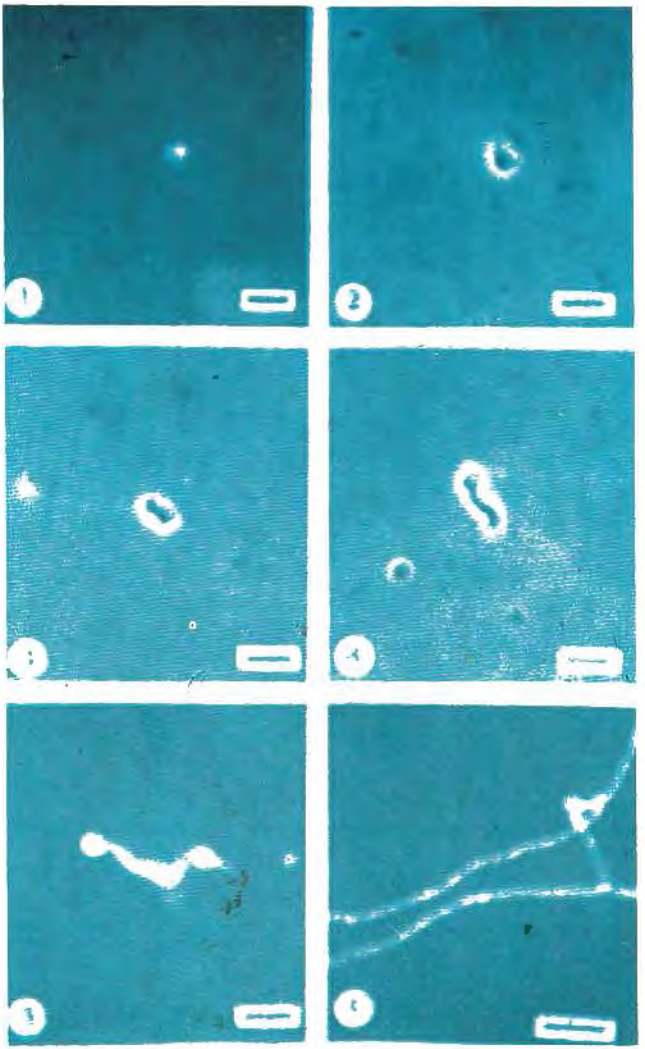
(1) Emergence of protoplast at the swollen hyphol tip in 0.6 M kcl after 30 min. digestion.

(2) Protoplasts liberated from the hyphol tip in 0.6 M $MgSO_4$ after 2 h digestion.

(3) Protoplasts emerging from subapical hyphol in 0.6 M kcl after 1 h digestion. The protoplasts are small and non-vacuolate.

(4) Highly vacuolate protoplasts liberated from the subapical zone in 0.6 M $MgSO_4$ after 3 h digestion.

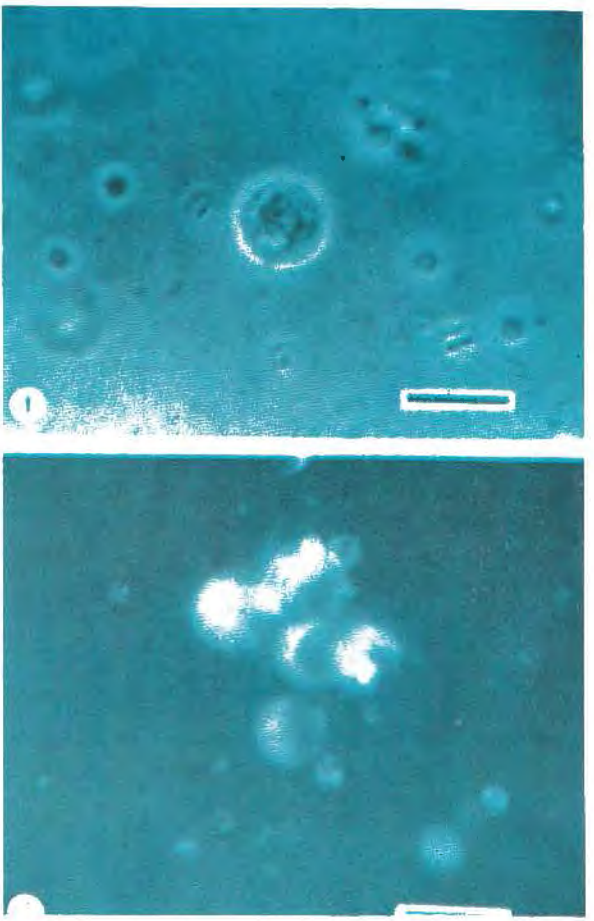
Bar markers represent 5μ.



Morphology of protoplast reversion during incubation in liquid culture at 30° C

(1) Uninucleate protoplast from 3 h digestion in kcl osmotic stabilizer
(2) Protoplast showing constricted appearance after 4 h digestion
(3) Budlike structure begin to appear after 5 h reversion
(4) Budlike structure elongate to form a normal hyphol after 6 h reversion
(5) A multinucleate hyphol originating directly from reverted protoplast after 8 h
(6) Normal hyphol develop and elongate to a multinucleate structure

Bar markers represent 5μ

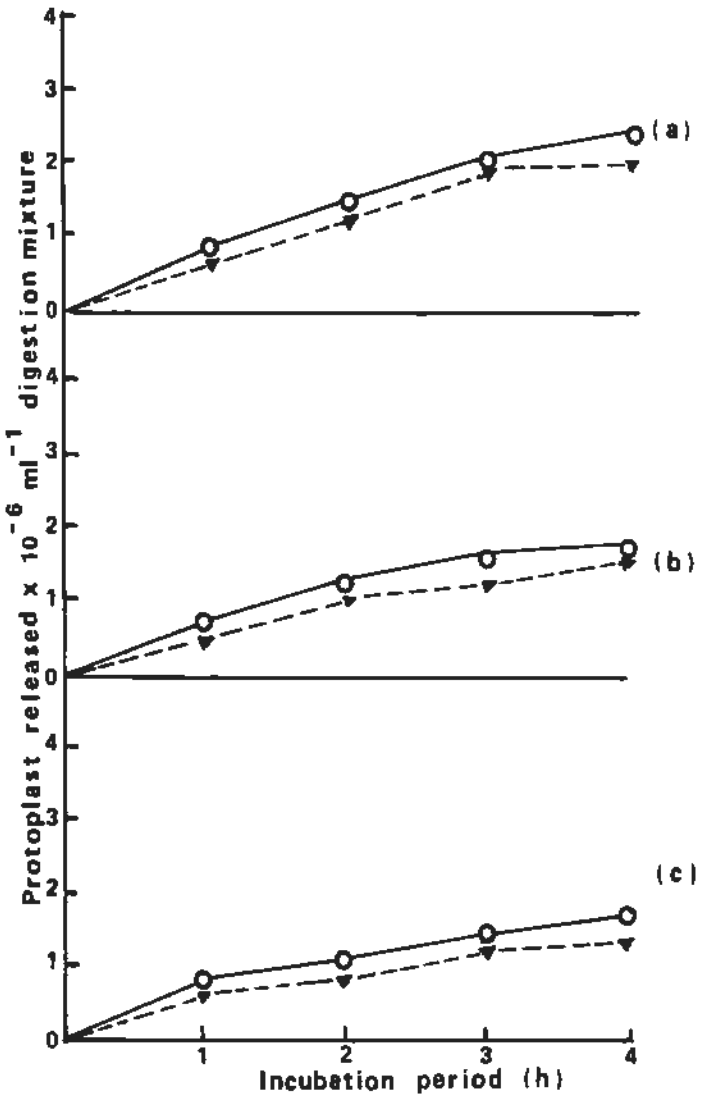


Morphology of large protoplasts after 24 h incubation in $MgSO_4$ stabilizer
Phase contrast microscope

(1) Highly vacuolate giant protoplasts. Unstained
(2) Highly vacuolate giant protoplasts
Fluorescent staining with chromomycin A_3.
(3) Fixed overnight with 4% glutaraldehyde

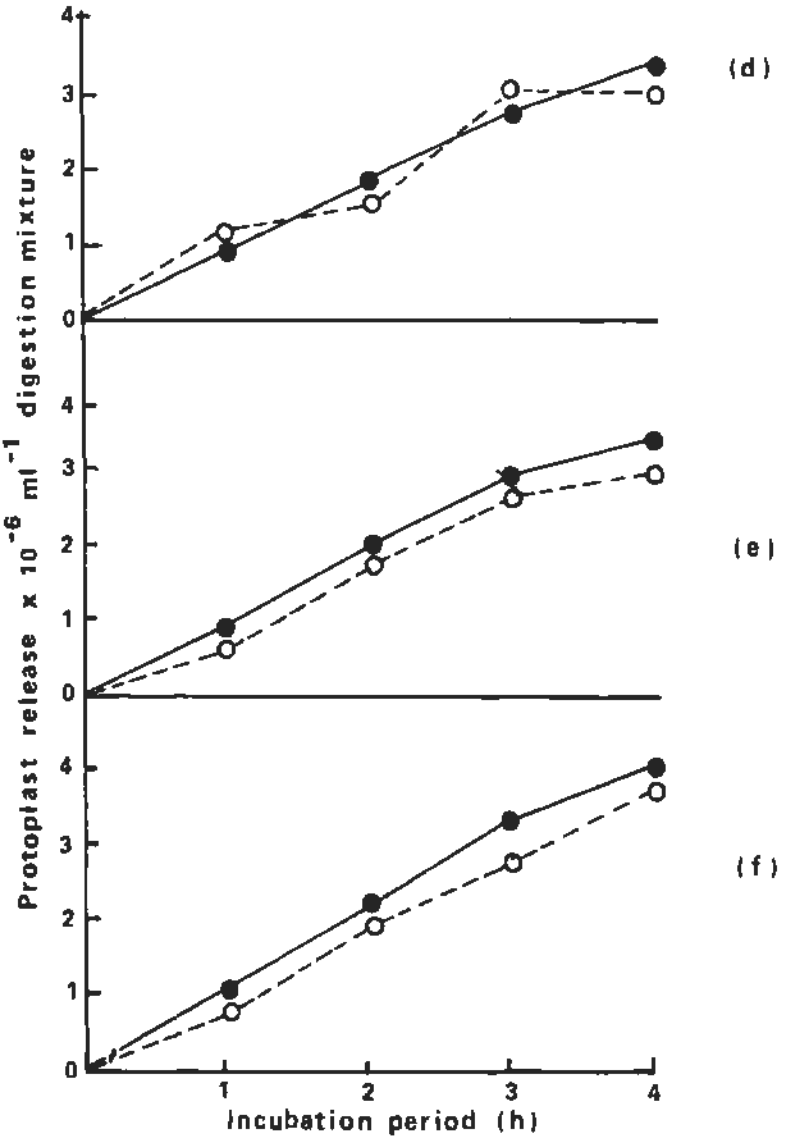
Bar markers represent 5μ.

Figure 1. Effect of medium supplement in Trichoderma narzanianum liquid culture (ten-fold concentrates) with 0.6 M $MgSO_4$ (●——) or with 0.6 M KCl (○— —) stabilizers on protoplast release.



(a) TLE + hyphal walls (5g. 1^{-1} dry weight)
(b) TLE + laminaria meal (5 g.1^{-1})
(c) TLE + chitin (5 g.1^{-1})

Figure 1. Effect of medium supplement in Trichoderma narzariarium liquid culture (ten-fold concentrates) with 0.6 M $MgSO_4$ (●——) as with 0.6 M KCl (○– –) stabilizers on protoplast yield.



(d) TLE + hyphal walls (5 $g.l^{-1}$ dry weight) + laminaria meal (5 $g.l^{-1}$)
(e) TLE + hyphal walls (5 $g.l^{-1}$ dry wt.) + chitin (5 $g.l^{-1}$)
(f) TLE + hyphal walls (5 $g.l^{-1}$ dry wt.) + laminaria meal (5 $g.l^{-1}$) + chitin (5 $g.l^{-1}$)

Table 1. Activities of chitinase and α— and β-glucanase in tenfold concentrate of 4 day culture filtrates from *trichoderma harzanum*.

Culture	*Chitinase*	*α-Glucanase*	*β-Glucanase*
	μ moles G/C Nac. min^{-1} .mg $protein^{-1}$*	*μ moles glucose min^{-1} .mg $protein^{-1}$*	*μ moles glucose min^{-1} .mg $protein^{-1}$*
TLE + Homogenized mycelium (100g. 1^{-1} fresh weight)	4.59 ± 0.05**	0.03 ± 0.02	2.17 ± 0.03
TLE + Laminaria meal (10g.1^{-1}) +Chitin (5g.1^{-1})	39.96 ± 0.10	0.07 ± 0.015	2.15 ± 0.081

* N — acetylglucosamine

** Standard deviation

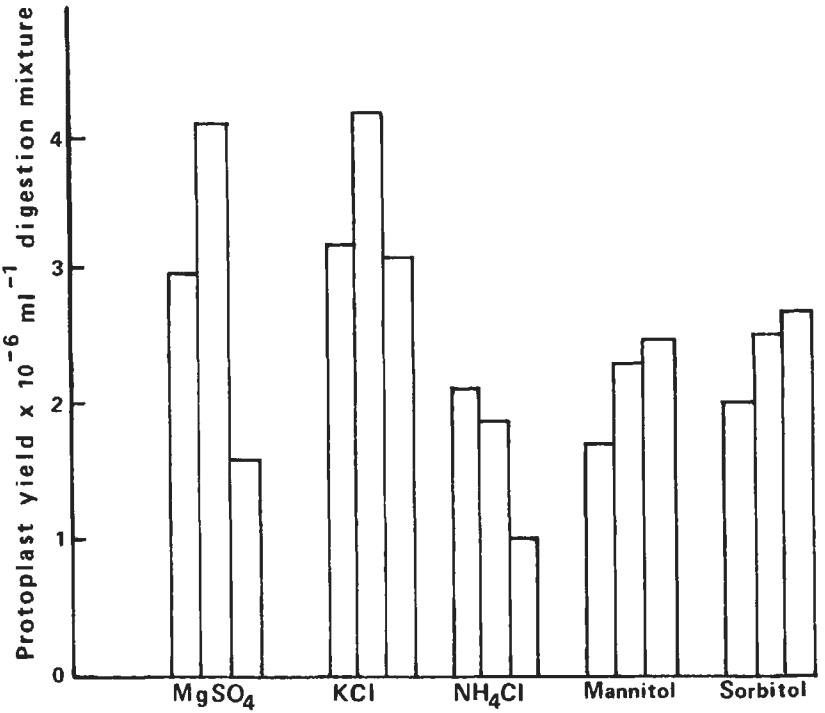


Figure 2. Comparison of protoplast yields using different osmotic stabilizers. The concentrations of stabilizer are 0.4 M, 0.6 M and 0.8 M from left to right. The digestions were carried out for 4 hr. at 27°C.

Effect of various stabilizers and their molarity.

Various compounds were tested for their effectiveness in supporting protoplast release and stability (Fig. 2). The relative density of the protoplasts varies, depending on the osmotic stabilizers used. Incubation with $MgSO_4$ or KCl gave more than 40% increase in yield compared to other stabilizers. In NH_4 Cl, mannitol, and sorbitol small non-vacuolate protoplasts were released in the early phases of digestion, but later several vacuolated protoplast appeared similar to the pattern of release in the KCl-stabilizer system.

$MgSO_4$ and KCl proved more effective than the sugar alcohols for this fungus. The use of these organic salts instead of organic compounds also prevented bacterial growth during incubation (10). $MgSO_4$ is preferred because most of the liberated protoplast developed large vacuoles and floated. This affords a convenient method for the recovery of these protoplasts from the residual hyphal fragments by slowspeed centrifugation. Protoplasts isolated with this stabilizer system were found to have a high reversion capability.

Effect of carbon source on reversion of protoplasts

The ability of protoplasts to regenerate a new cell wall and undergo reversion to the hyphal state was investigated by plating appropriate dilutions, after haemocytometer count, into hypertonic agar media. The presence of a carbon source is required for the reversion of protoplasts (Fig. 3). With asparagine as nitrogen source glucose, lactose, sucrose, fructose, maltose, arabinose and ribose generally increased the reversion rate of protoplasts. Stimulation of reversion by the polyols was lower compared to monosaccharides, dissaccharides and pentoses. The efficient stimulation of reversion by the disaccharide could be caused by the greater permeability of the protoplast or a greater rate of utilization and assimilation. The germination promoting action in some fungal spores has been suggested to be due to the presence of phosphorylase, B-fructofuramosidase and invertase activities (11, 12, 13) but no effort was made in the present study to identify these enzymes.

Effect of nitrogen source

A similar trend indicating an increase in the reversion frequencies of protoplasts was obtained when various nitrogenous compounds were added to the hypertonic agar medium. The results presented in Fig. 4 show that reversion is much better with the organic substrates. KNO_3 and NH_4 Cl promote less than 2% reversion. Without a nitrogen supplement in the medium, protoplasts do not revert. The reversion frequency obtained in *Aspergillus nidula** *s* (14) was lower at 10-30% than that reported for *Neurospora crassa (15) and Schizophyllum commune* (16). However, in *V. volvacea* the reversion frequency was exceedingly low and never greater than 4%. The inability of most protoplasts to revert may be due to the absence of nuclei (17) or the absence of other organelles in the cytoplasm (18).

Influence of different parameters on the fusion frequency

To determine the effectiveness of PEG as a fusogenic agent for *V. volvacea* protoplasts, sterile auxotrophic mutants which failed to produce sporophores in several conventional crossing experiments were fused. The criterion used to identify fused protoplasts was the formation of heterokaryons between nutritionally complementary auxotrophs growing on minimal medium. The fusion frequency (Ff) was expressed as the number of colonies on minimal medium (mmm) to the number of colonies developed on complete medium (mcm) after 2 days incubation at 37° C.

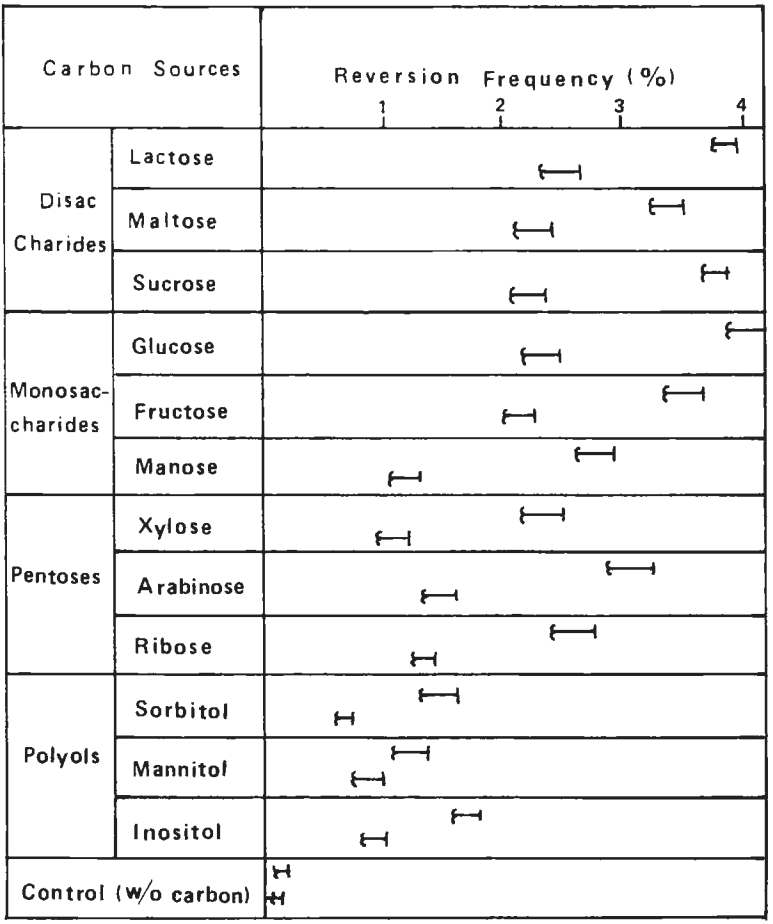


Figure 3. Effect of various carbon sources on the reversion of protoplasts. Protoplasts were isolated after 4 hr lytic digestion and incubated at 37°C for 2 days on 0.6 M $MgSO_4$ (||||) or 0.6 M KCl (:::) stabilized media.

$$Ff = \frac{\text{number of colonies on mmm}}{\text{number of colonies on mcm}} \times 100$$

Effect of PEG concentration

A solution containing 30% (w/v) PEG was found optimal in fusion and stability of protoplasts (Fig. 5). Most protoplasts were observed to burst at concentration lower than 20% especially

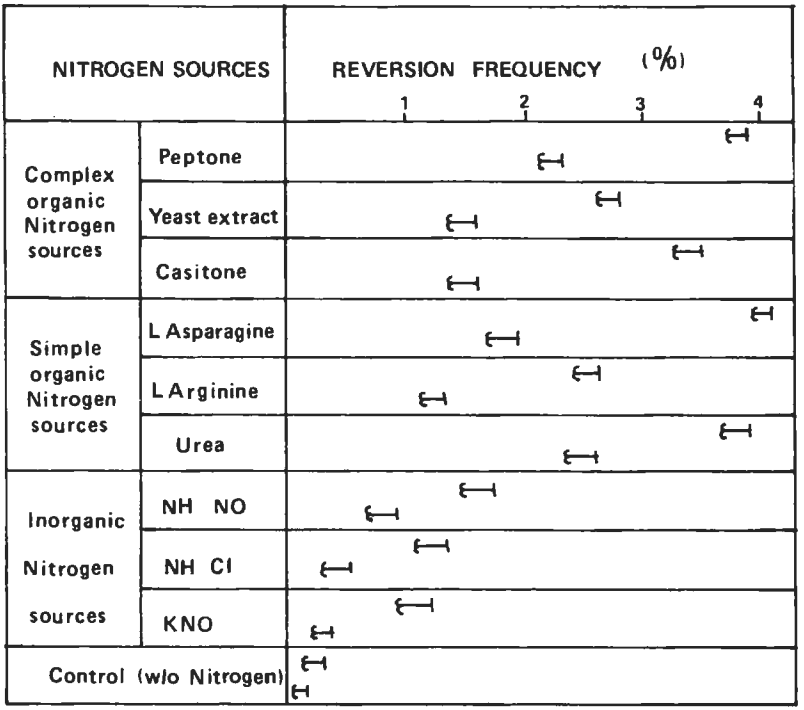


Figure 4. Effect of various nitrogenous sources on the reversion of protoplasts. Protoplasts were isolated after 4 hr lytic digestion and incubated at 37°C for 2 days on 0.6 M $MgSO_4$ (||||) or 0.6 M KCl (⋮⋮⋮) stabilized media.

when the amount of stabilizer was reduced. Above 40% PEG solution the protoplasts showed shrinkage probably due to the hypertonic conditions, but they normally regained their size after dilutions of PEG.

Effect of pH

There was an increased frequency with increasing pH and the highest yield of fusion was obtained at pH 8.0 (Fig. 6). Above this value heterokaryon formation was noticeably decreased. The effect of pH on fusion frequency was probably influenced by the presence of calcium ions from the fusion mixture containing $CaCl_2$.

Effect of temperature

Fusion of protoplasts initially took place at 10°C and there was a considerable increase with increasing temperature. The

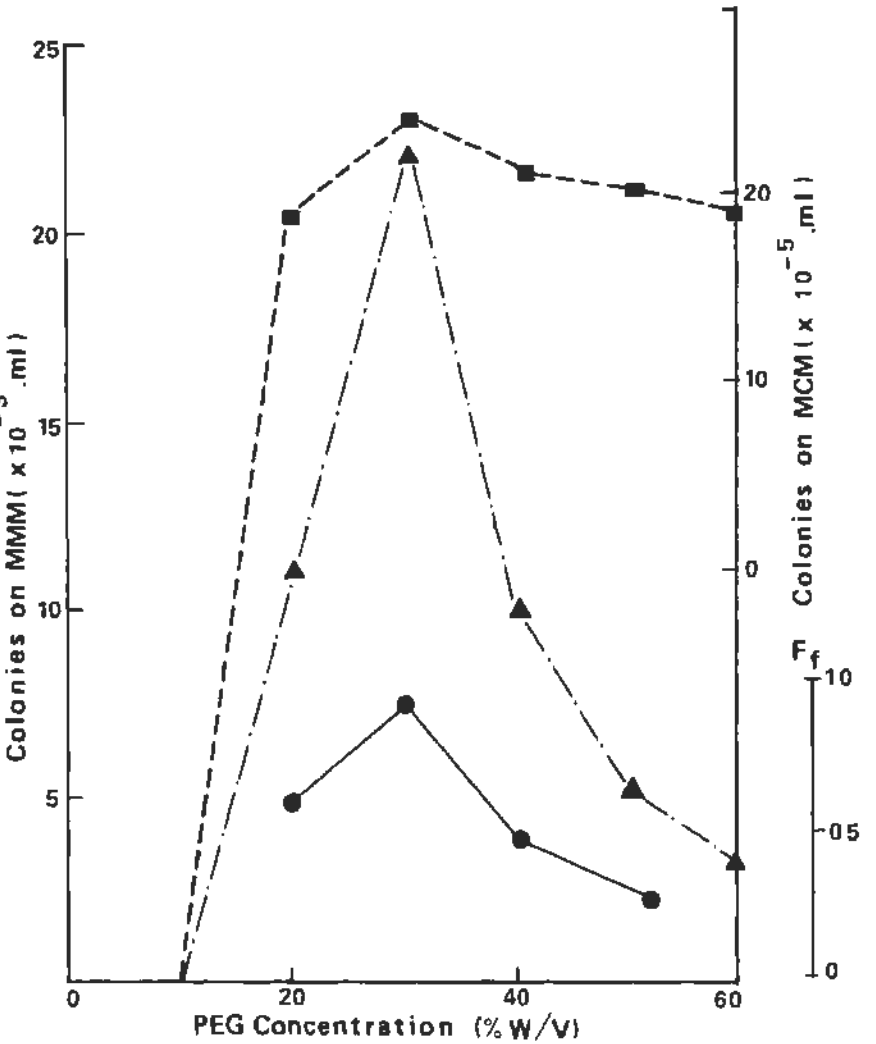


Figure 5. Effect of concentration of PEG on the fusion frequency (●——) of protoplast between Ade (R-type) x Leu (T-type) cross. Protoplasts were treated with different concentrations of PEG in 0.01 M $CaCl_2$ and 0.05 M glycine, pH 8 (30°C, 10 min). Heterokaryons and total number of viable protoplasts were counted in MMM (▲——) and MCM (■ — —) after 2 days at 30°C.

optimum temperature was found at 30°C (Fig. 7). The number of regenerating protoplasts decreased sharply above 40°C. This is probably due to reduction in viscosity of PEG solution at high temperature.

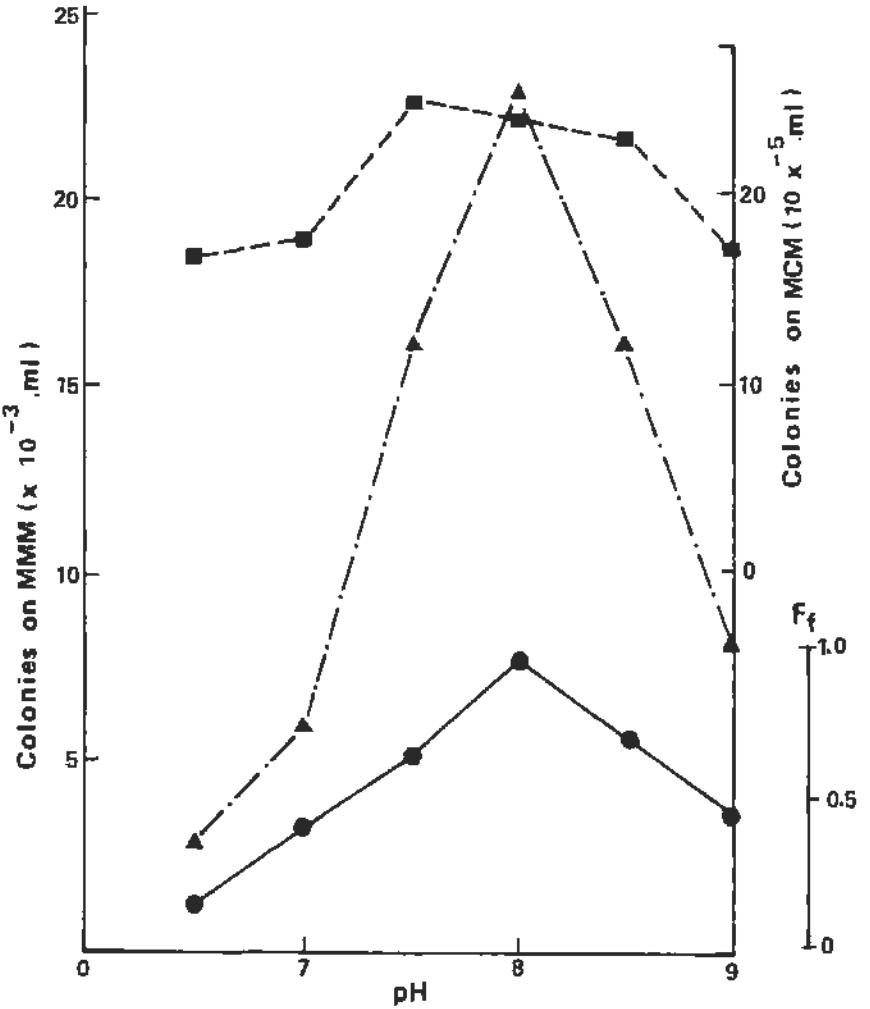


Figure 6. Effect of pH on the fusion frequency (●——) of protoplasts between Ade (R-type) x Leu (T-type) cross. Protoplasts were treated with 30% (W/V) PEG solution containing 0.01 M $CaCl_2$ and 0.05 M glycine. pH was adjusted with 0.01 M NaOH. Heterokaryons and total number of viable protoplasts were counted in MMM (▲—— ·) and MCM (■ — —) after 2 days at at 30°C.

Heterokaryon formation and fruiting between auxotrophic mutants using protoplasts fusion

No back mutation was found in any of the auxotrophs so the colonies recovered on mmm, following PEG treatment, were deemed to be heterokaryons. The frequency of heterokaryon

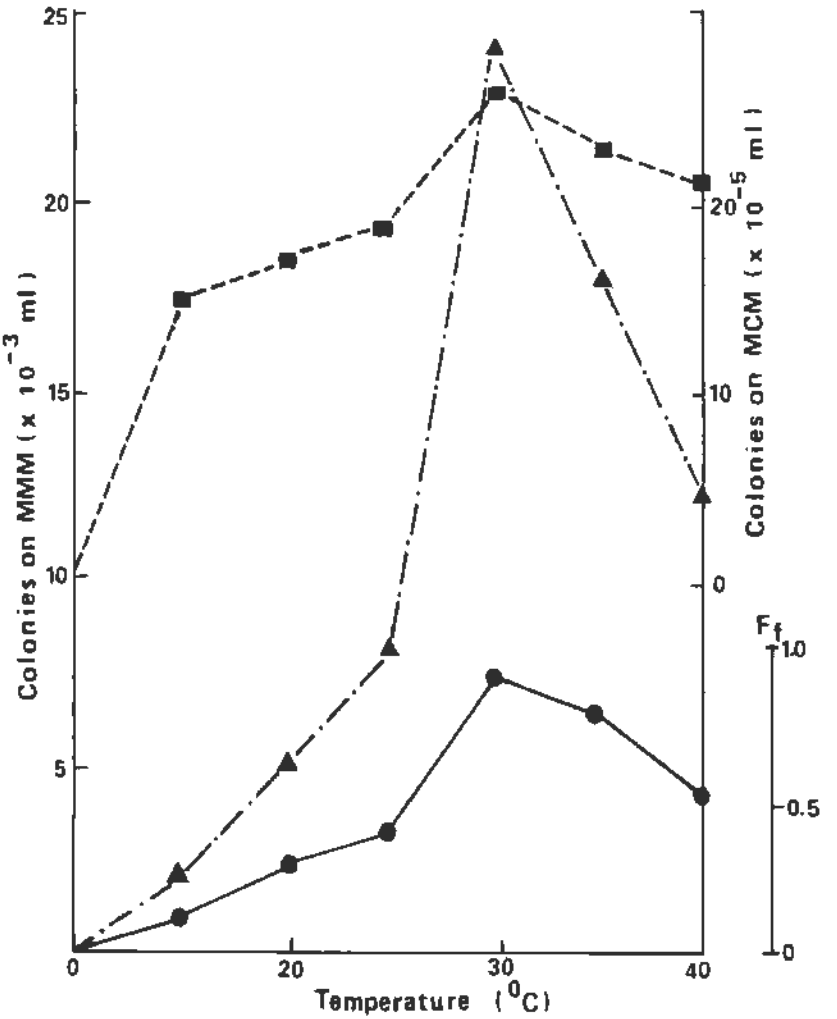


Figure 7. Effect of temperature on the fusion frequency (●——) of protoplasts between Ade (R-type) x Leu (T-type) cross. Protoplasts were washed with 0.6 M $MgSO_4$ and incubated at different temperatures. Heterokaryons and total number of viable protoplasts were counted in MMM (▲—— •) and MCM (■ — —) after 2 days.

formation was generally low (< 0.7%) and variable (Table 2). The heterokaryons were isolated and maintained on mmm. The colonies were irregular and morphologically different from the parental strains. Further evidence for heterokaryons was obtained when the fusion product was successfully fruited. Analysis of the single spore isolates from crosses 1 and 2 showed the presence of both recombinant classes, i.e. prototrophs and double auxotrophs.

Table 2. Frequencies of heterokaryon formation following protoplast fusion between antagonistic strains

Cross	*Protoplast mixtures*	*Heterokaryons (x $10^{-3}ml^{-1}$) on MMM*	*Colonies (x$10ml^{-6}$ $.ml^{-1}$) on MCM*		*Fusion Frequency*
		After PEG treatment	*Before PEG*	*After PEG*	
1	*Ade* (R-type) x *Leu* (T-type)	22	18.4	3.5	0.63
2	*Nia* (L-type) x *Met* (Q-type)	25.2	24.0	7.3	0.35
3	*Nia* (L-type) x *Arg* (D-type)	10.9	13.6	2.30	0.47
4	*Nia* (D-type) x *Ade* (R-type)	9.5	10.8	5.8	0.16

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