KOR. JOUR. MICROBIOL. Vol. 22, No. 2, 103~110, 1984

Intraspecific Protoplast Fusion in *Trichoderma koningii*

***Hong, Soon-Woo, Yung-Chil Hah, Hee-Moon Park, and Nam-Jin Cho**

Department of Microbiology, College of Natural Sciences, Seoul National University, Seoul, Korea

*Trichoderma koningii*의 種間 原形質體 融合에 대한 硏究

洪淳佑・河永七・朴喜門・趙南鎭

서울大學校 自然科學大學 微生物學科

ABSTRACT

The conditions for the protoplast fusion of auxotrophic mutants of *Trichoderma koningii* were determined. A preparation of commercial enzyme Driselase was used successfully to isolate protoplasts from the 18 hr old mycelium of *T. koningii*. The yields of protoplasts production were ranged from 0.3×10^8 to 2.5×10^8 protoplasts per mg of damp mycelium of various auxotrophic mutant strains. The regeneration frequencies from 9.3×10^{-3} to 2.0×10^{-1} were obtained when the protoplasts from auxotrophic mutants were plated on the malt extract medium containing 0.6M $MgSO_4$ and 2% agar, and the optimal concentration of PEG for protoplast fusion was 30%. Exposure of protoplasts to PEG for 10 min was found to be sufficient to induce high frequency heterokaryon formation. Optimal pH of fusion mixture was determined as 5.5, and 1 mM of calcium chloride in fusion mixture was found to be sufficient to enhance protoplast fusion frequency. Under optimal condition, the fusion frequency of the cross between protoplasts from various auxotrophic mutants were 1.6×10^{-2} and 4.1×10^{-2}.

INTRODUCTION

The imperfecti fungus, *Trichoderma koningii*, has potential applications in commercial processing of cellulose (Flickinger, 1980). These organisms have already been used in numerous studies on cellulose degradation (Halliwell and Riaz, 1970; Wood and McCrae, 1972; Halliwell and Griffin, 1973). However, the genetic study of these organisms has not been done because of its asexual nature. Their potential could be enhanced, if genetic method for producing strains with superior capacities were available. Recently, protoplast fusion has been considered to be a useful technique for studying basic and applied genetics of microorganisms for which the technique to exchange genetic materials has not been well developed (Peberdy, 1980), and cosiderable interests have been focused on the isolation of protoplasts and their use in fusion and transformation experiments (Ferenczy, 1981). However, there are only few reports on the isolation of protoplasts from industrially important fungus *Trichoderma* species (Benitez *et al.*, 1975; Picataggio *et al.*, 1983; Toyama *et al.*, 1983), and no reports on the fusion of protoplasts from *T. koningii*. We have already reported on the conditions for the isolation of protoplasts from the mycelium and conidiospores of *T. koningii* (Cho *et al.*, 1981a, 1981b; Park *et at.*, 1983), and on the electron microscopic observ-

* Author to whom all correspondence should be addressed.

ation on the protoplasting and regeneration mode of protoplasts from mycelium of *T. koningii* (Lim *et al.*, 1983). In this presentation we describe the conditions for fusion of protoplasts from the mycelium of *T. koningii*. We believe this to be the first original paper on the protoplast fusion of *T.koningii*.

MATERIALS AND METHODS

Organisms and culture

Trichoderma koningii ATCC 26113 and its auxotrophic mutants derived from the method as described below were used in this experiment. All the strains were cultured on malt extract medium containing 20g of malt extract (Difco), 1g of Bactopeptone (Difco) and 10g of dextrose per liter. Modified Mandel's medium (10g of dextrose, 2g of potassium phosphate, 1.4g of ammonium sulfate, 0.3g of magnesium sulfate, 0.3g of calcium chloride, 5mg of ferrous sulfate, 1.4mg of zinc sulfate, 1.56mg of manganese sulfate, and 2.0mg of cobalt chloride per liter) was used for the minimal medium (MM). For the regeneration complete medium (RCM) and regeneration minimal medium (RMM), malt extract and minimal medium supplemented with 0.6M magnesium sulfate were used repectively, and for the preparing regeneration agar medium, 2% agar was autoclaved separately to avoid acid hydrolysis.

Isolation of auxotrophic mutants

Conidiospore suspension of parental strains (1.0×10^7/ml) in 0.01% tween 80 was exposured to ultraviolet light (8~9erg/cm^2/sec) with shaking for 25 min to kill over 99% of the population. Auxotrophic mutants were enriched by the filtration method. The mutagenized conidiospores were suspended in the liquid minimal medium at a concentration of 10^6 per ml and incubated with shaking at 28°C. After 12 hrs of incubation, the culture was filtered through the sintered glass filter (40~60μm, KIMAX) to remove the germlings of parental type cells, and the ungerminated spores in the filtrate were collected by centrifugation and resuspended in the fresh minimal medium. The enrichment steps were repeated two or three times, and the spores were plated on the complete agar medium. Conidiospores from the colonies that appeared after incubation were plated to the fresh complete agar plates, and the resulting colonies were checked for growth on the minimal agar plates. The growth factors of mutants were determined by transferring them to various supplemented medium. In this experiment of mutagenesis, Oxgall (1.5%, w/v) and sodium deoxycholate (0.06%, w/v) were added to the solid medium for the restriction of the colonies. The list of various mutants derived from ultraviolet light treatment is shown in Table 1.

Table 1. Lineage of mutants derived from ultraviolet light mutagenesis

Strain	Parent strain	Genotype and phenotype
C-1	ATCC 26113	Chatreuse color conidia, Prototype
A-7	ATCC 26113	Arginine requiring
AF-1	ATCC 26113	Adenine requiring
ClUl	C-1	Chatreuse color conidia, Methionine requiring

Protoplast preparation and regeneration

The preparation and regeneration of protoplasts derived from the mycelium of *T. koningii* were performed by the methods described earlier (Cho *et al.*, 1981a, 1981b). Conidiospores (2.5×10^7) of each strain were inoculated into Erlenmyer flask (250ml) containing 50ml of the complete broth medium. Cultures were then incubated on a rotatory shaker at a speed of 200 rpm at 28°C for 18 hrs, and mycelia were harvested by centrifugation and then washed twice with the osmotic stabilizer (0.6M magnesium sulfate in 0.01M phosphate buffer, pH5.8), Pelleted mycelia were treated with Driselase (Kyowa Hakko Co. Tokyo, 1%, w/v) in the stabilizer

to give a final concentration 0.1mg of damp mycelia per ml of enzyme solution and then reacted for 3 hrs at 30°C with gentle shaking. Protoplasts were purified from the residual mycelial debris by filtration through the sintered glass filter and washed twice with stabilizer by centrifugation 700×g, at 4°C for 20min, and washed protoplasts were resuspended in the stabilizer and plated on the RCM or RMM for regeneration. The number of protoplasts was monitored with the aid of hemacytometer.

Fusion of protoplasts

The protoplasts from two parental strains were mixed (5.0×10^6 protoplasts, each) and sedimented by centrifugation. The pelleted protoplasts were resuspended in the minute volume of the remaining stabilizer by gentle shaking, and the protoplasts were treated with 1 ml of PEG (M.W. 6,000, Sigma) containing calcium chloride at 28°C. To examine the factors affecting protoplast fusion, the concentration of PEG (ranged from 10 to 40%, w/v), the pH of PEG solution (ranged from 3.5 to 10.5), and the periods of exposure to PEG (ranged from 10 to 90min) were tested. After incubation, the fusion mixture was serially diluted and spread on the RCM, and thereafter, regeneration frequency was determined by the number of colonies appeared on the RCM in relation to the number of protoplasts inoculated, and fusion frequency determined by the number of colonies appeared on the RMM in relation to the number of colonies appeared on the RCM plates.

RESULTS

Formation and regeneration of protoplasts

Under the conditions described, the formation of protoplasts from the mycelium of *T. koningii* ATCC 26113 was found to be linear for 3 hrs incubation, and after that, the rate decreased considerably and protoplast was highly vacuolated (see Plate 1) with prolonged incubation. The wild type hyphae from culture grown for 18 hrs yielded approximately 2.1×10^8 protoplasts per mg of damp mycelium, and the same level of protoplasts yield was obtained from the mycelia of the auxotrophic mutants except for the strain ClUl (Table 2). The regeneratien and spontaneous reversion frequency of the protoplasts from various auxotrophic mutants are shown in Table 2. The regeneration frequency on the RCM in relation to the number of protoplasts inoculated varied from 9.3×10^{-3} with the strain ClUl to 2.0×10^{-1} with the wild type strain. In the case of the strain A-7, none of viable colonies was developed on the RCM. Except for the wild type strain, all the mutant strains could not develop the viable colonies on the RMM in the range of below 10^{-6}. The real spontaneous reversion frequency of the ClUl may be much less than this value because plate counting was performed at the rate of $\times10^6$ or less.

Table 2. Formation, regeneration, and reversion frequency of protoplasts from auxotrophic mutants

Strain (genotype)	Yield of protoplast production ($\times10^8$)[a]	Regeneration frequency on RCM	Reversion frequency on RMM
ATCC 26113 (prototype)	2.1	2.0×10^{-1}	NT[b]
AF-1 (ade)	2.5	1.2×10^{-1}	4.6×10^{-7}
A-7 (arg)	2.0	ND[c]	1.1×10^{-8}
ClUl (met)	0.3	9.3×10^{-3}	2.8×10^{-6}

a) 1.0×10^8 protoplasts per mg of damp mycelium
b) Not tested
c) Not detected

Effect of PEG concentration on complementing heterokaryon formation

PEG and calcium chloride ions are widely used to induce fusion of protoplasts from many microorganisms. The effect of PEG concentration on fusion frequency is shown in Figure 1.

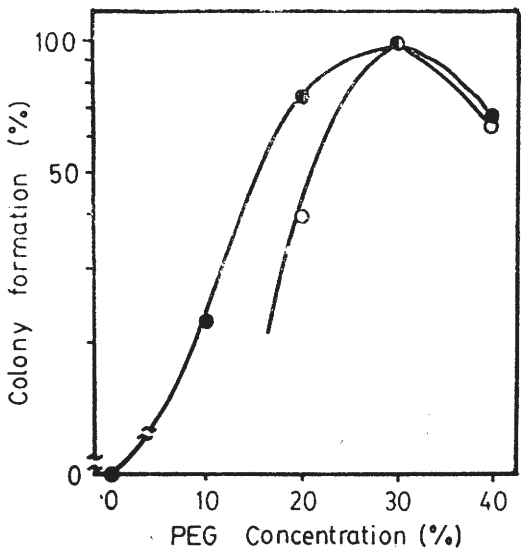

Fig. 1. The effect of polyethylene glycol concentration on the formation of viable colony on regeneration complete agar medium (●—●) and the formation of complementing heterokaryon on regeneration minimal medium (○—○). The average result obtained at the optimum polyethlene glycol concentration (30%) was taken as 100. The polyethlene glycol was treated for 30 min.

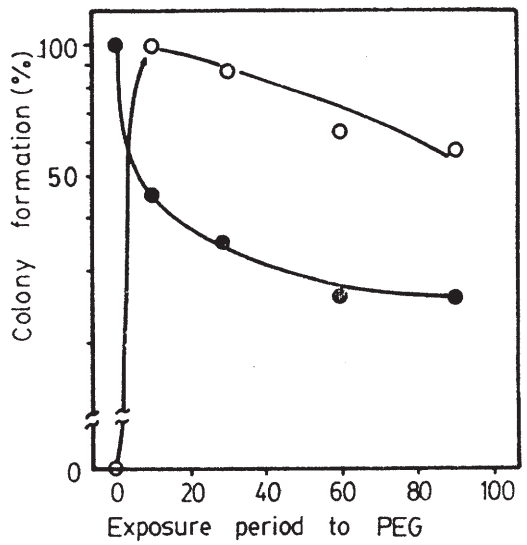

Fig. 2. The effect of the exposure period to polyethylene glycol on the formation of viable colony on regeneration complete agar medium (●—●) and the formation of complementing heterokaryon on regeneration minimal agar medium (○—○). The maximum value was taken as 100.

The protoplasts from the mutant strain A-7 and ClUl were mixed, treated for 30 min with varied concentration of PEG solution containing 10mM calcium chloride, serially diluted and spread on the RCM and RMM. The protoplasts from the wild type strain were also treated with varied concentration of PEG solution containing 10mM calcium chloride and plated on the RCM plates. After two or three days of incubation, the number of colonies developed on the RCM was counted. As shown in Figure 1, the fusion frequency and viable colony forming frequency were highest at the concentration of 30% PEG, concurrently.

Effect of periods of exposure to PEG on protoplast fusion

The effect of PEG treatment time on protoplast fusion was examined. Protoplasts from the wild type strain were treated with PEG(30%) containing 10mM calcium chloride at various duration, and thereafter, the protoplasts were plated on the RCM to determine the number of regenerating colonies. As shown in Figure 2, the number of regenerating colonies declined with a prolonged incubation with PEG solution. Under the same condition, the fusion frequency on the cross strain A-7 and strain ClUl was estimated, and found to decline similarly with a prolonged incubation with PEG; however, the rate of decrease was relatively slow. It was found that the fusion frequency was greatest at 10 min.

Effect of pH on protoplast fusion

To determine the optimal pH for protoplast fusion, the solution of 30% PEG 6,000 containing 10mM calcium chloride was prepared in various buffer system. These solutions were used for the fusion of protoplast from strain A-7 and strain ClUl(Table 3). The fusion mixture buffered with 50mM glycine (pH 5.5) was shown to have the highest fusion frequency. For subsequent experiments, the fusion mixture buffered

Table 3. Effect of pH on protoplast fusion frequency[a]

Buffer system of fusion mixture	Fusion frequency	% value
Citrate buffer pH 3.5	4.0×10^{-4}	2.7
Glycine buffer pH 5.5	1.5×10^{-2}	100.0
Phosphate buffer pH 7.5	3.8×10^{-3}	25.3
Tris-HCl buffer pH 9.0	2.6×10^{-3}	17.3
Carbonate buffer pH 10.5	3.0×10^{-4}	2.0

a) Protoplasts from the auxotrophic strain A-7 and ClUl were used in this fusion experiment, and the maximum value was taken as 100%.

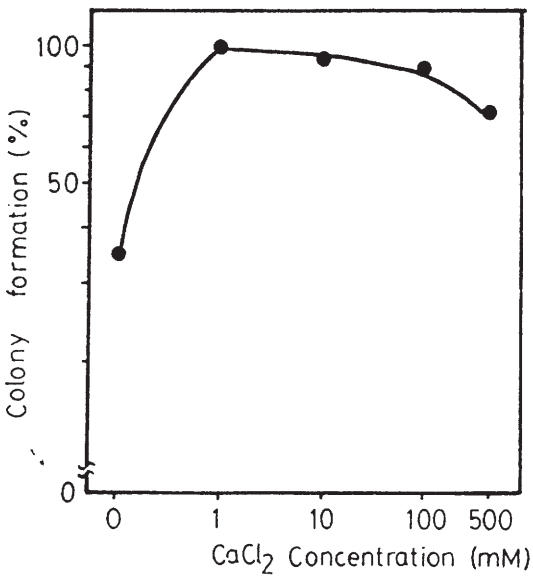

Fig. 3. The effect of $CaCl_2$ concentration on complementing heterokaryon formation. The average result obtained at the optimum $CaCl_2$ concentration (1mM) was taken as 100.

with glycine was used.

Dependence of calcium chloride concentration

The optimal calcium chloride concentration for protoplast fusion was examined by using 30% PEG 6,000 solution containing varied concentration of calcium chloride ranged from 0 to 500mM. As shown in Figure 3, the calcium chloride concentration over 1mM was sufficient to enhance the protoplast fusion; however, the fusion frequency decreased considerably at high concentration (500mM).

Complementation of auxotrophic mutants by protoplast fusion

The treatment of protoplasts from auxotrophic mutants with 30% PEG and 10mM calcium chloride in 50mM glycine(pH 5.5) led to fusion of protoplasts. The fusion frequency for complementing heterokaryons of the cross strain A-7 and strain AF-1 and the cross strain A-7 and ClUl were 4.1×10^{-2} and 1.6×10^{-2} respectively (see Table 4). For the control experiments, the protoplasts of two auxotrophic strains were mixed without PEG-$CaCl_2$-glycine mixture, and the protoplasts of a single strain mixed with fusion mixture. In both cases, no complementing prototrophic colonies were observed.

DISCUSSION

In the previous reports, we described the formation and regeneration of protoplasts from the mycelium of *T. koningii* ATCC 26113 (Cho *et al.*, 1981a, 1981b; Lim *et al.*, 1983). On the present report, we have described studies on the conditions of protoplast fusion. The commercial cell wall lytic enzyme, Driselase, has been used to prepare protoplasts from several fungal species(Picatassio *et al.*, 1983; Finkelman *et al.*, 1980; Gaupy and Ferve, 1982). The main protein components of Driselase are β-1,3-and β-1,6-glucanase. Protoplasts were successfully produced from the mycelium of *T. koningii* by using Driselase as a protoplasting agent. This result indicates that one of the cell wall components of *T. koningii* may be β-1,3-and β-1,6-glucan.

The regeneration frequency of protoplasts from the different strains varied from 9.3×10^{-3} to 2.0×10^{-1} under the conditions described. However, the protoplasts from mutant strain A-7 could not develop any viable colonies, perhaps due to the defects of cell wall sythesizing process by mutagenic treatment of ultraviolet light.

Table 4. Fusion frequency of the crosses between protoplasts from various auxotrophic mutants

Cross(genotype)[a]	Fusion frequency
A-7(arg) × A-7(arg)	ND[b]
ClUl(met) × ClUl(met)	ND[b]
AF-1(ade) × AF-1(ade)	ND[b]
A-7(arg) × AF-1(ade)	4.1×10^{-2}
A-7(arg) × ClUl(met)	1.6×10^{-2}

a) Abbreviations: ade, adenine; arg, arginine; met, methionine
b) Not detected

The maximum frequencies of heterokaryon formation and viable colony formation were obtained at a 30% concentration of PEG. These results suggest that incubation with PEG solution at the concentration below 30% may cause marked lysis of protoplasts and that over 30% marked aggregation of protoplasts. The frequency of protoplast fusion and viable colony formation decreased with a prolonged incubation with PEG solution. From this result, it is suggested that the longer the period of exposure to PEG solution was, the larger number of protoplasts aggregated together.

The optimal pH of fusion mixture for heterokaryon formation was 5.5, and the fusion frequency was enhanced by the addition of $CaCl_2$. A similar effect was shown by the $CaCl_2$ concentration ranged 1 to 100mM, however, the fusion frequency of protoplasts was dropped at a high concentration of $CaCl_2$ (500mM).

The results in Table 4 show that complementing heterokaryons could be obtained by the protoplast fusion. The spontaneous reversion frequencies of protoplasts from various auxotrophic mutants ranged from 2.8×10^{-6} to 1.1×10^{-8}, thus the probability that the complementing colonies may result from spontaneous reversion is excluded.

The results reported here show that the protoplast fusion technique will be a very effective tool for the genetic studies of cellulolytic imperfecti fungus *T. koningii*.

Isolation and genetic analysis of the stable complementing hybrids by the protoplast fusion of *T. koningii* and interspecific protoplast fusion between *T. koningii* and *T. reesei* have been done in this laboratory, and the results will soon be published.

摘 要

*Trichoderma koningii*로부터 유도된 영양요구성 돌연변이주간의 原形質體 融合을 위한 條件을 조사하였다. 18時間 배양한 *T. koningii*의 균사체에 Driselase를 처리하여 原形質體를 추출할 수 있었다. 原形質體의 生成量은 각 영양요구성 돌연변이주의 菌絲體 1mg당 0.3×10^{8}~2.5×10^{8}수준이었으며, 0.6M $MgSO_4$와 2% agar가 첨가된 malt extract배지상에서의 原形質體 還元率은 9.3×10^{-2}~2.0×10^{-1}수준이었다. 原形質體 融合을 위한 polyethylene glycol (PEG)의 최적농도는 30%였으며, PEG를 10분간 처리하였을때, 높은 빈도의 heterokaryon 形成이 가능하였다. 融合 혼합물의 최적 pH는 5.5이었으며, 1mM의 $CaCl_2$가 첨가되었을때 融合率이 진작되었다. 최적조건하의 原形質體 融合率은 1.6×10^{-2}와 4.1×10^{-2} 수준으로 나타났다.

REFERENCES

Benitez, F., S. Rams and I. Garcia Acha, 1975. Protoplasts of *Trichoderma viride*; formation and regeneration, *Arch. Microbiol.* 103, 199-203.

Cho, N.J., Y.H. Rhee and S.W. Hong, 1981a. Formation of protoplast from *Trichoderma koningii*, *Kor. J. Microbiol.* 19(4), 186-191.

Cho, N.J., H.M. Park and Y.H. Rhee, 1981b. Protoplast reversion of *Trichoderma koningii*, *Kor. J. Microbiol.*, 19(4), 192-198.

Ferenczy, L., 1981. Microbial protoplast fusion. In Genetics as a tool in microbiology (Ed., S.W. Clo-

ver and D.A. Hopwood) pp. 1-34 Cambridge University Press

Finkelman, H.A., J.E. Zajc and A. Vardanis, 1980. New method of producing protoplasts of *Aureobasidium pullulans*. *Appl. Environ. Microbiol.* 32:923-925.

Flickinger, M.C., 1980. Current biological research in conversion of cellulosic carbohydrates into liquid fuels; How far have we come?. *Biotech. Bioeng.* 22 (suppl. I):27-48.

Gaupy, D. and M. Ferve, 1982. Protoplast production from *Saprolegnia monoica*. *Microbios.* 34:89-98.

Halliwell, G. and M. Griffin, 1973. The nature and mode of action of the cellulolytic components C_1 of *Trichoderma koningii* on native cellulose. *Biochem. J.* 135:587-594.

Halliwell, G. and M. Riaz, 1970. The formation of short fibers from native cellulose by components of *Trichoderma koningii* cellulase. *Biochem. J.* 116: 35-42.

Lim, H.M., H.M. Park, Y.C. Hah and S.W. Hong, 1983. Electron microscopic study of protoplasts released from mycelium of *Trichoderma koningii*. *Kor. J. Electron Microscopy.* 13:49-61.

Park, H.M., S.W. Hong and Y.C. Hah, 1983. Isolation of protoplasts from conidiospore of *Trichoderma koningii*. *Kor. J. Microbiol.* 21:213-220.

Peberdy, J.F., 1980. Protoplast fusion-a tool for genetic manipulation and breeding in industrial microorganisms. *Enzyme Microbiol. Technol.* 2:23-29.

Picataggio, S.K., P.H.J. Schamhart, B.S. Montenecourt and D.E. Eveleigh, 1983. Spaeroplast formation and regeneration in *Trichoderma reesei*. *Eur. J. Appl. Microbiol.* 17:121-128.

Toyama, H., A. Shinmyo and H. Okada, 1983. Protoplast formation from conidia of *Trichoderma reesei* by cell wall-lytic enzymes of a strain of *Trichoderma viride*. *J. Ferment. Technol.* 61:409-411.

Wood, T.M. and S. McCrae, 1972. The purification and properties of the C_1 components of *Trichoderma koningii* cellulase. *Biochem. J.* 128:1183-1192.

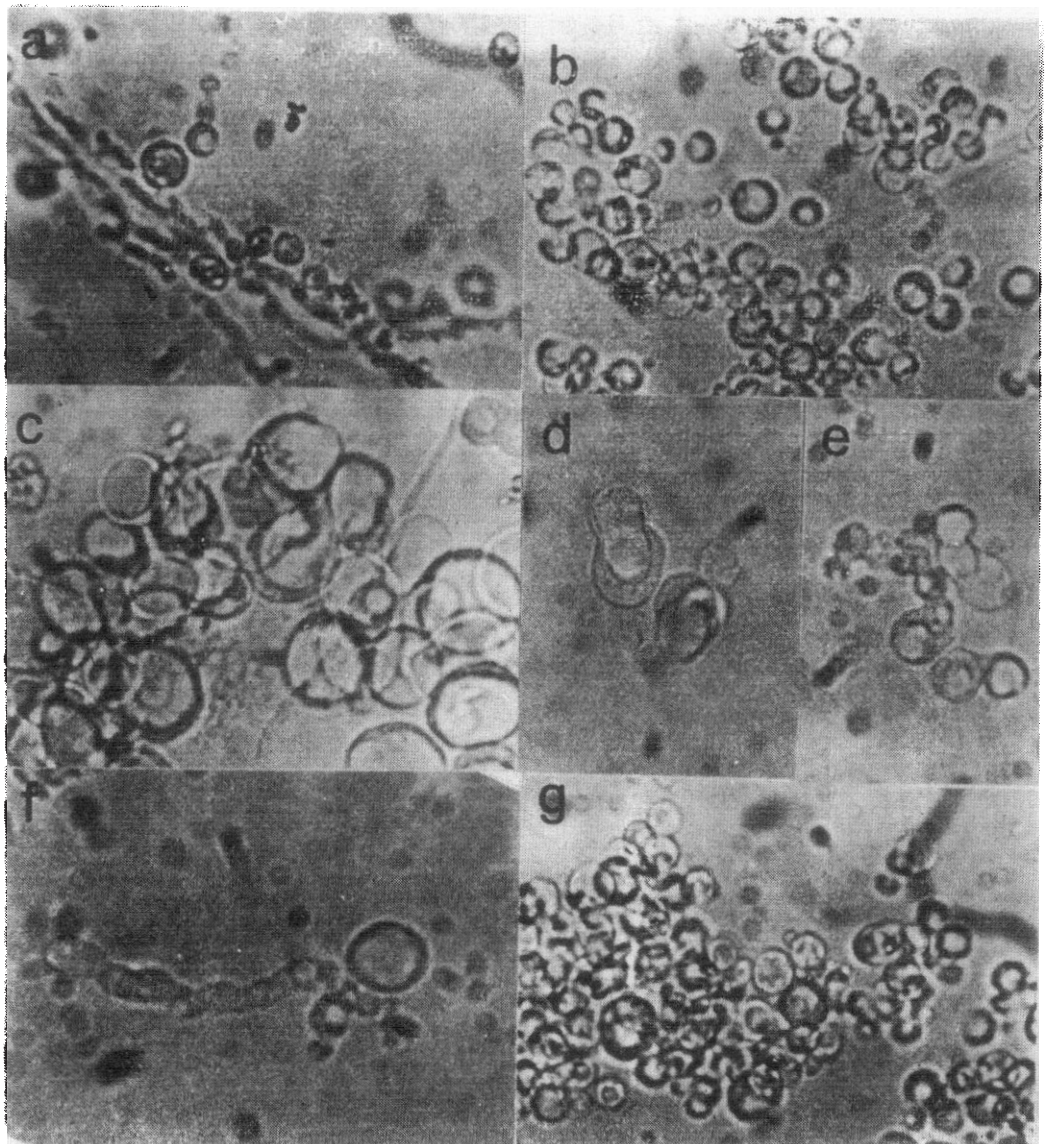

Plate 1. Formation, regeneration and aggregation of protoplasts from *Trichoderma koningii*. a. protrusion of protoplasts from the mycelium by the treatment of Driselase. (1%) ; b. purified protoplasts slightly vacuolated; c. highly vacuolated protoplasts with prolonged incubation; d, e, yeast-like form of regenerating protoplasts; f, aberrant-tube formation of regenerating protoplasts; g. large protoplasts aggreagates formed after PEG treatment. All the detailed modes of formation and regeneration of protoplasts were reported previously (Cho *et al.*, 1981b; Lim *et al.*, 1983).