THE KOREAN JOURNAL OF MYCOLOGY
Vol. 21, No 4, p323-331 December 1993

Printed in S. KOREA

Intergeneric protoplast fusion between *Gliocladium virens* and *Trichoderma harzianum*

Pyung-Gyun Shin and Moo-Je Cho*

Plant Molecular Biology and Biotechnology Research Center,
Department of Biochemistry, Gyeongsang National University,*
Chinju 660-701, Korea

*Gliocladium virens*와 *Trichoderma harzianum*의 屬間 原形質體融合

신평균 · 조무제*

경상대학교 식물분자생물학 및 유전자조작 연구소
*경상대학교 생화학과**

ABSTRACT: The protoplast formation and intergeneric protoplast fusion between *Gliocladium virens* and *Trichoderma harzianum* were attempted to obtain fusants. Protoplast formation was the most effective when the strains were treated with concentration of 5 mg/ml of Novozyme 234 and Cellulase at 25℃ for 3 hours in phosphate buffer, pH 6.5, supplemented with 0.6 M sorbitol as osmotic stabilizer. Auxotrophic mutants of *G. virens* G88 did not grow in minimal medium and benomyl resistant *T. harzianum* T95 from wild types, however, was selected by treatment with UV light as genetic marker to isolate fusants. When the intergeneric protoplast fusion between *G. virens* G88 and *T. harzianum* T95 was carried out using 30% PEG 4000 containing 10 mM $CaCl_2$ and 50 mM glycine (pH 8.5) as fusogenic agent at 25℃ for 10-15 min, the fusion frequency was 0.8×10^{-4}. Fusants obtained from intergeneric protoplast fusion were spontaneously segregated into various strains by continous culture on complete medium. Several intergeneric hybrids were classified into three types: parent-like hybrids, segregants, and recombinants.

KEYWORDS: Biological control, *Gliocladium*, *Trichoderma*, Intergeneric protoplast fusion

Introduction

Biological control of plant pathogens, which utilizes one or more biological processes to lower inoculum density of the pathogens or to reduce its disease producing activities, has been a major subject in the area of plant pathology. This has been the case in the hope of reducing the harzards of various fungicides and other agrochemicals on the ecosystem(Mukerji and Gary, 1988).

Gliocladium virens has been effectively used for control of several soilborne plant pathogen, especially in the damping-off diseases of zinnia, cotton, and cabbage caused by *Pythium* spp. and *Rhizoctonia solani* in nonsterile soilless mix by producing an antibiosis(Howell, 1982; Lumseden and Locke, 1989). Antibiosis can be a principal component of the biological control mechanism by *G. virens*. However, understanding the mechanism is complicated by the possible presence of several metabolites such as gliotoxin, gliovirin, gliocladic acid, heptalidic acid, viridin, viridiol, and valinotricin etc., which are produced by *G. virens*(Bryan, 1944; Bryan and McGowan, 1945; Howell and Stipanovic, 1983; 1984; Itoh *et al*, 1980).

Trichoderma spp. closely related to the *G. virens* are known to produce extracellular enzymes and secondary metabolites, some of which may be in-

*Corresponding author

volved in the biological control of other phytopathogenic fungi(Papavizas, 1985). It was known that *Trichoderma* spp. interact with other phytopathogenic fungi in a variety of ways. *Trichoderma* spp. coil around the pathogens to penetrate their hypae to other fungi and subsequently kill the culprit by producing toxic metabolites which come from the hypae(Cutler *et al*, 1986; Hadar *et al*, 1979; Well *et al*, 1988). Extracellular cell wall degrading enzymes such as 1, 3-β-glucanase and chitinase were also known to be involved in having detrimental effects by the fact that increased enzymatic activities occurred at the point where *Trichoderma harzianum* attached to *R. solani* by coiling(Elad *et al*, 1982; Harmon and Hadar, 1983; Ridout *et al*, 1986).

Another interesting aspect of the *Trichoderma* spp. is the plant growth-promoting effect. Suslow (1982) reviwed several potential mechanisms in the growth-promoting effect on plants. He argued that the plant could benefit either from the growth-stimulating substances such as hormones or other growth factors or nutrients produced by the fungus. Plant growth might also be indirectly stimulated to take up nutrients by the presence of the microorganisms in the soil. On the other hand, growth responses induced by *Trichoderma* spp. could appear to be due to both the control of minor pathogens and production of growth-regulating factors(Baker, 1988).

Genetic manipulation of biocontrol agents offers a possible approach to improving their potential for the integrated biological control of plant pathogens. Intergeneric protoplast fusion and the incorporation of prokaryotic DNA into eukaryotes will definitely be an asset in evolving the desired disease lines in a highly controlled manner. Recently, intergeneric protoplast fusion has been carried out between *Aspergillus niger* and *Trichoderma viride. Saccharomycopsis fibuligera* and *Yarrowia lipolytica*, and *Trichoderma reesei* and *Penicillium funiculosum*(Kirimura *et al*, 1988; Nga *et al*, 1992; Pham and Halos, 1990). Although protoplast fusion between *Trichoderma* spp. or *Gliocladium* spp. was completed, fusion between intra- and interspecific *Trichoderma* spp. appeared to have a very low compatibility with each other(Stasz *et al*, 1988).

Here, we report the intergeneric protoplast fusion between *Gliocladium virens* and *Trichoderma harzianum* to obtain a hybrid which can carry the beneficial traits from both parents.

Materials and Methods

Fungal species and culture media

The wild type fungal species, Gliocladium virens and *Trichoderma harzianum*, and their mutant auxotrophs, *G. virens* G88 and *T. harzianum* T95, were described in elsewhere(Bae *et al*, 1990). All strains were cultured on complete medium (CM) containing yeast extract 10 g, glucose 30 g, casamino acids 5 g, peptone 4 g, sucrose 20 g, KH_2PO_4 0.46 g, K_2HPO_4 0.46 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g per liter. Minimal medium(MM) containing glucose 20 g, KH_2PO_4 0.46 g, K_2HPO_4 1 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g per liter. The regeneration complete medium(RCM) and regeneration minimal medium(RMM) were prepared by adding 0.6 M sorbitol to CM and MM, respectively. For solidifying regeneration medium, 0.75% softagar was overlayed with mixture of protoplast.

Protoplast formation and regeneration

Protoplasts were produced from mutants of *T. harzianum* and *G. virens* as modified method of Anne *et al.*(1974). Protoplasts were released by incubating of CM-grown mycelium treated with cell wall lytic enzyme containing 0.6 M sorbitol and 10 mM phosphate buffer(pH 6.5) for 3 h at 30 ℃ onto CM plate in petri dish. Protoplasts were purified from the residual mycelial debris by filtration through the sintered glass filter(porosity 1) and washed twice with 0.6 M sorbitol by centrifugation at 100xg for 10 min. The washed protoplasts were resuspended in the 0.6 M sorbitol and plated on the RCM or RMM for regeneration. The number of protoplasts was counted under light microscope using haemocytometer.

Isolation of auxotrophic mutants

Mutagenesis and filtration enrichment of *G. virens* and *T. harzianum* were based on the method

developed for *Aspergillus nidulans*. Vegetative spores of parental strains in 10 mM phosphate buffer(pH 6.8) were exposed to UV light(8^{-10} erg/cm^2/sec) with stirring for 1 to 30 min to kill over 99% of the population. The treated spores were lied under the dark condition for 2 h. To inhibition of back mutation or reversion, the mutagenized suspensions were diluted into 10^3, 10^4, 10^5 /m*l* and incubated for 3-4 days at 30℃ with shaking at 120 strokes/min in minimal medium. The germinated colonies were removed by filtering the suspension through sintered glass filter(porosity 1). The filterred suspension were then plated on complete medium including 0.05% Triton X-100 to limit the size of colonies. The putative auxotrophs were characterized by modification of Holiday's method(1956).

Protoplast fusion

Protoplasts isolated from *T. harzianum* T95 and *G. virens* G88 were fuged using polyethylene glycol(PEG) in the similar method to that described by Ferenczy *et al.*(1975). The isolated protoplasts from *T. harzianum* T95 and *G. virens* G88 were mixed about 5×10^8 /m*l* and centrifuged at 700xg for 10 min. The pelleted protoplasts were resuspended in 1 m*l* of a prewarmed polyethylene glycol 4000(PEG, sigma) containing $CaCl_2$ and glycine. It was adjusted to pH 8.0 with 10 mM NaOH, and incubated at 30℃ for 15 min. The incubated fusion mixture was serially diluted and spread onto RMM supplement 10 μg/m*l* benomyl with 0.75% top agar. The fusion frequency was determined from the number of colonies apppeared on the RMM and RCM plates.

Selection of fusants

The generated colonies onto hypertonic medium were selected. The colonies picked off with a cocktail stick were transfered into a minimal medium and incubated for 2-7 days at 25℃. The selected colonies were cultured continously for maintaining genetic stability and analyzed various morphology using the descriptive terminology of Stalpers (1978). And also production materials of fusants and parent were detected on TLC plates.

Results and Discussion

Various factors on protoplast formation

Cell wall lytic enzymes: To find suitable enzymes, commercially available enzyme preprations were screened for lytic activity against *T. harzianum* mycelia. As shown in Table 1, chitinase was not effective to release protoplast, while Cellulase Onozuka R-10 was slightly effective. Novozyme 234 and Cellulase Onozuka R-10 were the most effective, yielding 0.8×10^6 protoplasts per m*l* after 3 h at 25℃. This high efficiency of Novozyme 234 and Cellulase Onozuka R-10 for protoplast release seems to be due to the fact that the cell wall of *Trichoderma* contains β-1, 3-glucan and cellulose as main cell wall components,and Novozyme 234 contains a number of enzyme, i.e., chitinase, β-1, 3-glucanase, and protease(Hamlyn *et al*, 1981). Novozyme 234 was added at four different concentration of 3, 5, 10 and 15 mg/m*l*, and reacted at 25℃ for different periods from 1 to 4 h. In the combination of Novozyme 234 and Cellulase Onozuka R-10, the optimum protoplasts were obtained by addition about 1:1 at 5 mg enzyme concentration per m*l* for 3 h at 25℃. The number of protoplasts increased with increasing concentration of the lytic enzymes, but too high concentrations were harmful, resulting in lysis of protoplasts soon after their appearance, probably due

Table 1. Comparison of different commercially-available cell wall degrading enzymes for the release of protoplasts.

Enzymes*	Yield of protoplast($\times10^6$ /m*l*)	
	G. virens G88	*T. harzianum* T95
Novozyme 234	1.0	0.6
Chitinase	0.0	0.0
Cellulase Onozuka	0.3	0.1
Novozyme 234+ Cellulase Onozuka**	1.2	0.8

*Concentration of enzyme was treated 5 mg/m*l* for 3 hours at 25℃.

**Novozyme 234 (5 mg/m*l*) and Cellulase Onozuka (5 mg/m*l*) were mixed 1:1 (v/v).

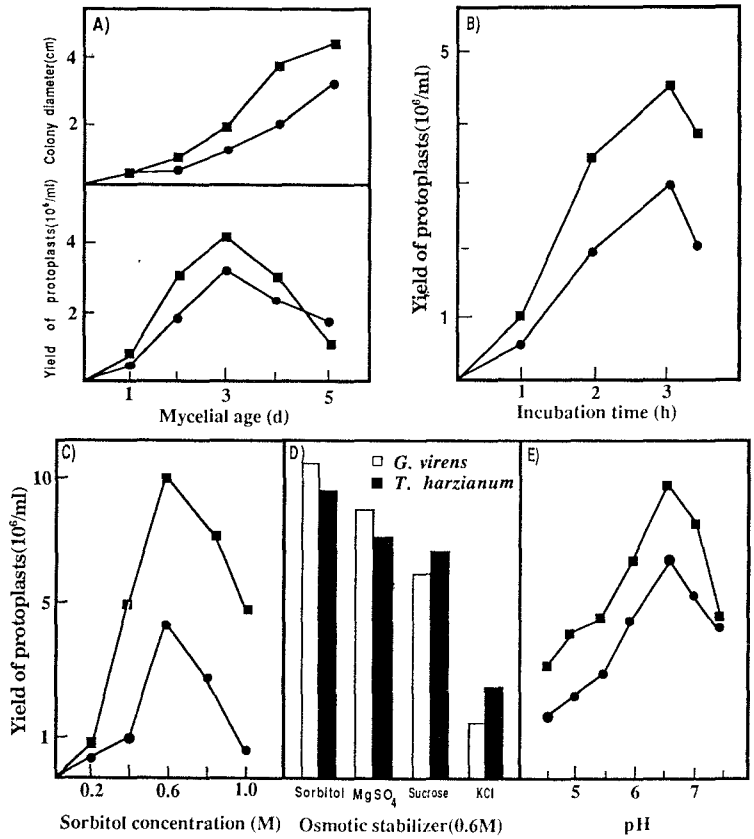

Fig. 1. Effect of various factors on protoplast formating of *G. virens* G88 (■) and *T. harzianum* T95 (●). A, Mycelial age; B, Incubation time (h); C, Sorbitol concentration (M); D, Osmotic stabilizer (0.6 M); E, pH.

to the high levels of proteinase in the digestive mixture.

Mycelial age: As shown in Fig. 1-A, the growth stage of mycelium clearly effected the number of protoplasts release. Large number of protoplasts were obtained at 3 d old mycelium which contained 2.2 cm of colony diameter. Older culture(4 d) was increased colony diameter, but was less effective for protoplast release. Use of mycelia older than 3 d was increased colony diameter but resulted in a significant decrease in protoplast yield. The efficiency of the protoplast formation inducing systems during the aging process of the mycelium could be due to alterations of the ratio and/or the texture of the cell wall components, influencing the susceptibility to the cell lytic enzyme. On the other hand, partial autolysis of the cell wall or the involvement of endogenous autolytic enzyme could contribute to the high number of protoplasts. Further decomposition of the cell wall and the cytoplasmic membrane probably might explain the decrease of the number of osmolabile bodies produced from mycelium of still older culture. This result corresponds with those of Peberdy *et al*,(1977), and Picataggio *et al*,(1980) who reported that the protoplast yield was the highest when the cultures were used at exponential growth phase.

Osmotic stabilizers and pH: As osmotic stabilizers for protoplast release, the following compounds of 0.6 M sorbitol, $MgSO_4$, sucrose or KCl were added to 0.1 M phosphate buffer(pH 6.5). As shown in Fig. 1-D, sorbitol was better protective effect than other stabilizer. The optimum molarity

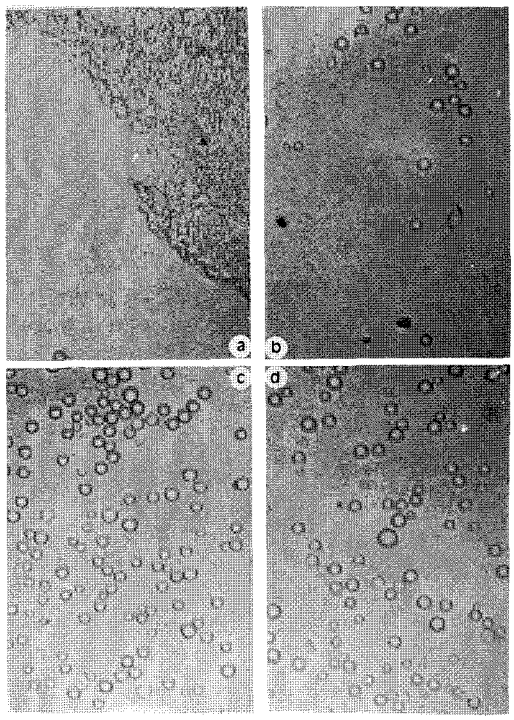

Fig. 2. Time-course release of protoplasts with the treatment of Novozyme 234.
a) Release of protoplasts from hyphae tips after 1 h (a), 2 h (b), 3 h (c) and 4 h (d) incubation at 25℃ with Novozyme 234 (5 mg/ml) in 0.6 M sorbitol as an osmotic stabilizer.

of sorbitol to stabilize protoplasts during release was, irrespective of the lytic enzyme, observed to be 0.6 M(Fig. 1-C). The protoplast yield resulted in a loss of protoplasts due to lysis in the hypertonic medium when the sorbitol concentrations were inhibitory to protoplast release and the liberated protoplasts shrank by the high osmotic pressure. pH for protoplast release was adjusted to pH in the range 4 to 8 with 0.1 M phosphate buffer and it was obtained that the maximum yield of protoplast was determined using 0.1 M phosphate buffer, pH 6.5, containing 0.6 M sorbitol(Fig. 1-E). No mycelia was digested at below pH 4 and protoplasts burst soon after emergence at above pH 8.

Incubation time: The reaction of protoplast release was carried out at 25℃ for 1-4 h on reciprocal shaker with 120 stroke/min. As shown in Fig. 1-B, the highest yield of protoplasts was obtained by incubating at 25℃ for 3 h. After 1 h incubation, some hyphal tip became swollen and protoplasts emerged by an extension of cytoplasm at the digested tips or in their close vicinity. Early protoplasts, protoplasts released in the 2 h incubation, were small spherical bodies, nonvacuolated and homogenous maximum, and protoplasts after 4 h incubation, were decreased and also size was large (Fig. 2). Similar differences in size between "early" and "late" protoplasts were observed by Gibson and Peberdy(1972). And size increased presumably due to the enlargement of the vacuole by osmosis and also by the biosynthetic activity during the incubation in the lytic enzyme solution as assumed from the continuing nuclear division in the protoplasts. Regeneration of protoplast was dependent upon the exposure time of the mycelia to the lytic enzyme. Higher regeneration frequency was obtained from protoplast released by short time of hydrolytic treatment, but longer period of incubation resulted in low regeneration frequency due to the demage of protein or lipoprotein components of the plasma membrane by proteolytic enzymes contaminated in the lytic enzyme preparation.

Selection of mutants from *T. harzianum* and *G. virens*

Filtration enrichment method for identification of biochemical mutants was used. As shown in Fig. 3, the highest number of *G. virens* auxotrophs was isolated after 8 min exposure at a 10% survival rate by treatment of UV light. It has also been shown to cause structural changes in chromosomes and also 10% of the surviving population of UV treated conidia carried chromosome aberrations. Kafer(1975) also reported that UV light may cause chromosomal translocations. As shown in Table 2, the selection of mutants was decided to set up fusion using the strains valuable in order to produce recombinants with at least two markers which could be valuable for future use. Unfortunately, attempts to obtain cross-fertilizable mutants in these fungi failed, but *G. virens* G88 did not grow in MM and *T. harzianum* T95 of benomyl resistane were selected.

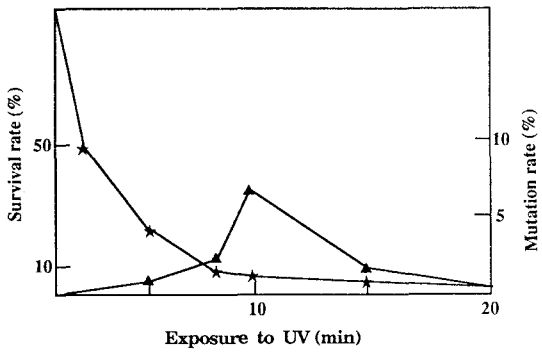

Fig. 3. Effect of UV-irridiation on the survival (★) and mutation rate (▲) for *G. virens* G88. Vegetative spores were mutagenized by UV-irridiation (254 nm) for 1 to 30 min at 1 cm distance and then incubated at 30℃ in minimal medium containing 0.01% Tween 80. The germinated colonies were removed by filtration. The ungerminated spores were plated on complete medium and the colonies which grow in complete medium but can not grow in minimal medium were selected as mutants.

Table 2. A list of fungicide resistances and auxotrophic mutants derived from ultraviolet light mutagens.

Strains	Genetic marker	Fungicides*	
		Benomyl	Iprodione
G. virens			
Wild type	Prototroph, green	S	R
G88	Non-growth in MM, green	S	S
T. harzianum			
Wild type	Prototroph, yellow	S	R
T95	Prototroph, yellow	R	R

*Concentration of fungicides was 10 μg/ml; R means resistant and S means sensitive.

Various factors on protoplast fusion

Influence of PEG concentration: PEG and calcium chloride are widely used to stimulate fusion of protoplasts in various organisms. The protoplast from the mutant of *T. harzianum* T95(benomyl resistance) and *G. virens* G88(non-growth in MM) were mixed, and treated with various concentration of PEG solution containing 10 mM $CaCl_2$ in 0.1 M phosphate buffer(pH 6.5), and spread on RCM and RMM. After 4 day incubation, the number of colonies regenerated on RCM and RMM is counted. As shown in Fig. 4, the highest fusion frqueney was obtained at PEG 4000. To stabilize most of the protoplasts at least 20% PEG 4000 was needed, because in the absence of an additional stabilizer protoplasts burst in PEG solutions lower than 20%. Solutions containing 30% PEG 4000 stabilized with the smallest reduction in the number of protoplasts. PEG solutions at 40% or higher were very hypertonic condition and caused shrinkage of the protoplasts.

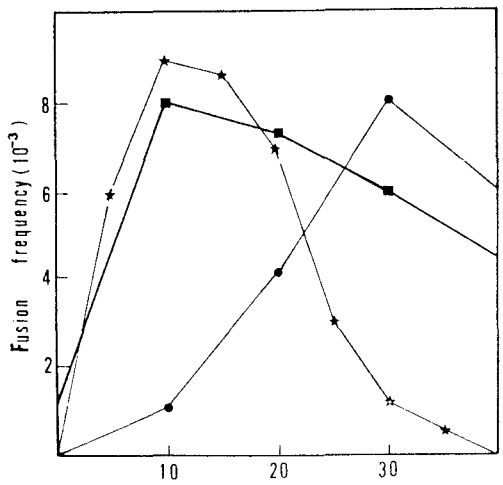

Fig. 4. Effect of various factors on the intergeneric protoplast fusion of *G. virens* G88 and *T. harzianum* T95.
●, PEG concentration (%, w/v) containing 10 mM of $CaCl_2$; ★, Exposure time (min) in 30% of PEG 4000; ■, $CaCl_2$ concentration (mM) with 30% of PEG 4000 for 10 min.

Influence of exposure time to PEG: The influence on aggregation of protoplasts occurred immediately after PEG was spreaded over the surface of the protoplasts. As shown in Fig. 4, the increase of fusion during 10-15 min may have been necded for complete coating of the protoplasts by PEG or for the reaction of all the fusogenic factors with the protoplast surface. This supposition was affirmed by the fact longer incubation did not increase in fusion frequency. The mechanism by which PEG induces membrane fusion is not known yet,

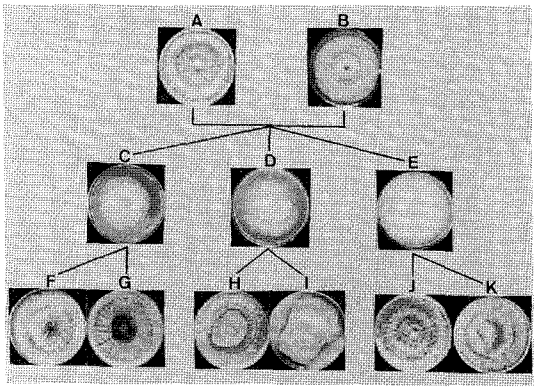

Fig. 5. Morphology of intergeneric fusants and segregants of *G. virens* G88 and *T. harzianum* T95.
A, *G. virens* G88; B, *T. harzianum* T95; C, D, and E, Cell fusants and segregants; F, G, H, I, J, and K, Cell fusants and segregants in continous culture.

but some suppositions have been given. Kao and Michaylak(1975) assumed that PEG molecules have hydrogen-bonds with water molecules, proteins, carbohydrates and phospholipids of the membranes whereby PEG might act as a molecular bridge between the surface of adjacent membranes of tightly-packed protoplasts. Removal of PEG during the washing process could result in disturbance and redistribution of electric charges between adjacent membranes resulting in membrane fusion. Because it is important for fusion that membranes can approch each other close enough, reduction of the water concentration at the protoplast membrane(Wallin *et al*, 1974) or the decrease of the surface potential of the membrane caused by PEG molecules also might be important in protoplast aggregation and memebrane fusion(Maggio *et al*, 1976).

Influence of calcium chloride concentration: Fusion occured after treatment of protoplasts with aqueous PEG solutions at glycine buffer(pH 8.0) without any addition of salt or sugar. Ca^{++} promoted the highest level of fusion at glycine buffer (pH 8.0). The optimal calcium chloride concentration for protoplast fusion was examined by using 30% PEG 4000 solution containing various concentration of calcium chloride ranged from 0 to 100 mM. As shown in Fig. 4, the highest level of fusion was observed when calcium chloride concentration was 10 mM. Calcium ions promote fusion, probably because they can neutralize negatively-charged groups. In addition, they might form bridges between the negatively polarized groups of the membranes and PEG, thus facilitating cellular aggregation. Furthermore, binding of Ca^{++} to phospholipids may allow other phospholipids to form fluid clusters, facilitating membrane fusion (Ahkong *et al*, 1975). If K^{+}, Na^{+} or Mg^{++} are present, in addition to Ca^{++}, they could come into competition with Ca^{++} for binding sites at the membranes, diminishing the number of Ca^{++} bound. Conceivably, this would decrease the effect of Ca^{++}, as reflected in the fusion yield.

Analysis of products and morphological features from fusants

Fusants that appeared on RMM were picked up and cultivated on MM plates. The fusion frequency was 6.2×10^{-4}. So far, we obtained 5 fusants, which are classifiable into three types according to their morphologies as shown in Fig. 5. As to the first type, 17 fusant strains formed colonies like those of *G. virens* G88 on MM and CM. They grew as well as the prototrophic parental strains were slight green those of *G. virens* G88. This type of fusant strins appeared distictly in continous culture. From these results, these fusant strains were assumed to be aneuploids. As to the second type, 30 fusant strains formed colonies showing mixed morphologies, between those of *T. harzianum* T95 and *G. virens* G88. The fusant strains of the second type were judged to be heterokaryons from the fellowing observations; when cultivated on CM, these strains formed predominantly green conidia of *G. virens* G88 in the central area and yellow conidia of *T. harzianum* T95 type in the peripheral area. To determine stability, fusant strains cultivated continously on CM at 25 ℃. From these results, these fusant strains were segregated and reverted into two parental strains. As to the third type, colonies of 7 fusant strain were completely different from that when the prototrophic parental strains were cultivated together.

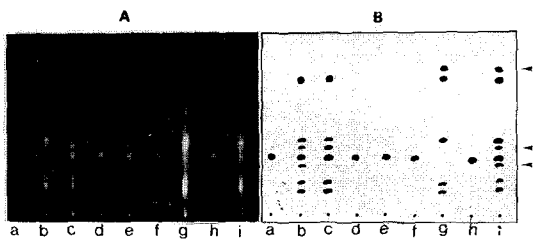

Fig. 6. Characterization of intergeneric fusants by the detection of fluorescent antagonistic compounds.
A) TLC chromatogram; B) Drawing of characteristic fluorescent compounds; TLC chromatogram was visualized by UV-transilluminator at 254 nm. a to f and i, Fusants of *G. virens* G88 and *T. harzianum* T95; g, *T. harzianum* T95; h, *G. virens* G88.

They grew more rapidly than the prototrophic parental strains and were stable in continous culture.

Comparison of production materials of fusants and parents detected on TLC plates. As shown in Fig. 6., the i fusant strain produced heterokaryon compounds. And this fusant strain was segregated in continuous culture(Fig. 5-H and I). The c fusant strain producing both the prototrophic parental strains and new fluorescent compounds was assumed to be heteroploid(Fig. 5-J and K). The a, b, c, d, and f fusant strains were produced *G. virens* G88 -like compounds(Fig. 5-F and G). From the above results, the protoplast fusion technique is useful for the construction of intergeneric hybrids between *Gliocladium* and *Trichoderma*, and that the exchange of genetic materials and taxonomical studies of imperfecti fungi can be done with the protoplast fusion.

摘 要

*G. virens*와 *T. harzianum*의 원형질체 형성 및 속간 원형질체 융합에 미치는 여러 요인의 영향과 이들 융합체의 특성을 조사하였다. 속간 원형질체 융합을 위하여 원형질체 형성은 삼투압 조절제인 0.6 M sorbitol을 함유한 0.1 M phosphate buffer(pH 6.5)에 Novozyme 234와 Cellulase를 1:1 비율로 3시간 동안 처리하였을 때 원형질체 형성이 가장 효과적이었다. 융합체를 선별하기 위한 유전적 지표로는 UV 처리에 의하여 최소배지에는 성장하지 못하는 *G. virens* G88과 benomyl 저항성을 나타내는 *T. harzianum* T95를 선발하였다. 속간 원형질체 융합 실험에는 10 mM $CaCl_2$를 함유한 glycine buffer(pH 8.5)에 융합제인 30% PEG 6000를 첨가한 용액을 25℃에서 10-15분간 처리했을 때 가장 높았으며, 융합효율은 0.8×10^{-4}였다. 선발된 융합체는 완전배지에서 계속 계대배양을 하여 유전적으로 안정한 세가지 유형의 융합체를 얻었으며 양친과 닮은 유형, 양친의 두 형질을 가진 유형 및 재조합된 유형으로 나타났다.

Acknowledgment

This work was supported in part by a research grant of Korea Research Foundation(1987-1989).

References

Anne, J., Eyssen, H. and de Somer, P. 1974. Formation and regeneration of *Penicillium chrysogenum* protoplasts. *Arch. Microbiol.* **98**: 159-166.

Ahkong, Q. F., Tampson, W. and Lucy, J. A. 1975. Promotion of cell fusion by divalent cation ionophores. *Nature* **256**: 208-209.

Bae, Y. S., Kim, H. G. and Park, C. S. 1990. An improved method for rapid screening and analysis of root colonizing ability of biocontrol agent. *Kor. J. Plant Pathol.* **6**: 325-332.

Baker, R. 1988. *Trichoderma* spp. as plant-growth stimulants. *CRC Critical Reviews in Biotechnology* **7**: 97-106.

Bryan, P. W. 1944. Production of gliotoxin by *Trichoderma viride. Nature* **154**: 667-668.

Bryan, P. W. and McGowan, J. C. 1945. Viridin: a highly fungistatic substance produced by *Trichoderma viride. Nature* **151**: 144-145.

Cutler, H. G., Cox, R. H., Crumley, F. G. and Cole, P. D. 1986. 6-Pentyl-α-pyrone from *Trichoderma harzianum*: Its plant growth inhibitory and antimicrobial properties. *Agri. Biol. Chem.* **50**: 2943-2945.

Elad, Y., Chet, I. and Henis. Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum. Can. J. Microbiol.* **28**: 719-725.

Ferenczy, L., Kevei, F. and Szegedi, M. 1975. High-frequency fusion of fungal protoplasts. *Experientia* **31**: 1028-1030.

Gibon, R. K. and Peberdy, J. F. 1972. Fine structure

of protoplasts of *Aspergillus nidulans*. *J. Gen. Microbiol.* **72**: 529-538.

Hadar, Y., Chet, I. and Henis, Y. 1979. Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum*. *Phytopathol.* **69**: 64-68.

Hamlyn, P. F., Bradshow, R. E., Mellon, F. M., Santiago, C. M., Wilson, J. M. and Peberdy, J. F. 1981. Efficient protoplast isolation from fungi using commercial enzymes. *Enzyme Microb. Technol.* **3**: 321-325.

Harmon, G. E. and Hadar, H. 1983. Biological control of *Pythium* species. *Seed Sci. Technol.* **11**: 893-906.

Holiday, R. 1956. A new method for the identification of biochemical mutants of microorganisms. *Nature* **178**: 987.

Howell, C. R. 1982. Effect of *Gliocladium virens* on *Pythium ultimum, Rhizoctonia solani,* and damping-off of cotton seedlings. *Phytopathol.* **72**: 496-498.

Howell, C. R. and Stipanovic, R. D. 1983. Gliovirin, a new antibiotic *Gliocladium*. *Can. J. Microiol.* **29**: 321-324.

Howell, C. R. and Stipanovic, R. D. 1984. Phytotoxicity to crop plants and herbicidal effects on weeds of viridiol produced by *Gliocladium virens*. *Phytopathol.* **74**: 1346-1349.

Itoh, Y., Kodama, K., Furuya, K., Takahashi, S., Heneishi, T., Takiguchi, Y. and Araj, M. 1980. A new sesquiterpene antibiotic heptelidic acid: Producing organisms, fermentation, isolation and characterization. *J. Antibiot.* **33**: 468-473.

Kafer, E. 1975. Reciprocal translocation and translocation disomics of *Aspergillus* and their use for genetic mapping. *Genetics* **79**: 7-30.

Kao, K. N. and Michaylak, M. J. 1974. A method for high-frequency intergeneric fusion of plant protoplasts. *Planta* **115**: 355-367.

Kirimura, K, Imura, M., Kato, Y., Lee, S. P. and Uasi, S. 1988. Intergeneric protoplast fusion between *Aspergillus niger* and *Trichoderma viride*. *Agri. Biol. Chem.* **52**: 1327-1329.

Lumseden, R. D. and Locke, J. C. 1989. Biological control of damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* with *Gliocladium virens* in soilless mix. *Phytopathol.* **79**: 361-366.

Maggio, B., Ahkong, Q. F., and Lucy, J. A. 1976. Polyethylene glycol, surface potential and cell fusion. *Biochem. J.* **158**: 647-650.

Mukerji, K. G., and Gary, K. L. 1988. Biocontrol of plant diseases vol. I, II., CRC Press, Florida.

Nga, B. H., Abu Bakar, F. D., Loh, G. H., Chiu, L. L., Harashima, S., Oshima, Y. and Heslot, H. 1992. Intergeneric hybrids between *Saccharomycopsis fibuligera* and *Yarrowia lipolytica*. *J. Gen. Microbiol.* **138**: 223-227.

Papavizas, G. C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology, and potetial for biocontrol. *Ann. Rev. Phytopathol.* **23**: 23-54.

Peberdy, J. F., Eyssen, H. and Anne, J. 1977. Interspecific hybridisation between *Penicillium chrysogenum* and *P.cyanneofulvum* following fusion. *Mol. Gen. Genet.* **157**: 281-284.

Pham, L. J. and Halos, S. C. 1990. Intergeneric protoplast fusion of *Trichoderma reesei* RUT C-30 and *Penicillium funiculosum* Thom MG-171 for improved cellulase production. *Enzyme Engineering* **10**: 575-581.

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

Ridout, C. J., Coley-Smith, J. R. and Lynch, J. M. 1986. Enzyme activity and electrophoretic profile of extracellular protein induced in *Trichoderma* spp. by cell walls of *Rhizotonia solani*. *J. Gen. Microbiol.* **132**: 2345-2352.

Stalpers, J. A. 1978. Identification of wood-inhibiting Asphyllophorales in pure culture. *Studies in Mycology, Baarn.* **16**: 1-248.

Stasz, T. E., Harmon, G. E. and Weeden, N. F. 1988. Protoplast preparation and fusion in two biocontrol strains of *Trichoderma harzianum*. *Mycologia* **80**: 141-150.

Suslow, T.V. 1982. Role of root-colonizing bacteria in plant growth. In Mount, M. S. and Lacy, G. H. (eds). *In Phytopathogenic Prokaryotes, chap.* **8**., Academic Press, New York.

Wallin, A., Glimelins, K. and Eriksson, T. 1974. The induction of aggregation and fusion of *Daucus carota* protoplasts by polyethylene glycol. *Z. Pflanzenphisiol.* **74**: 64-80.

Well, H. D., Bell, D. K., and Taworski, C. A. 1972. Efficacy of *Trichoderma harzianum* as a biocontrol for *Sclerotium rolfsii*. *Phytopathol.* **62**: 442-447.