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Optimization of formation and regeneration of protoplasts from biocontrol agents of *Trichoderma* species

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The optimal conditions necessary for a large yield and a high frequency of regeneration of protoplasts isolated from the biocontrol agents *Trichoderma koningii* and *T. harzianum* were investigated. Protoplast yields were 1.2×10^8/ml from *T. koningii* and 6×10^7/ml from *T. harzianum* when 20-h mycelial culture was treated with a lytic enzyme solution containing Novozym 234 (15 mg/ml), sucrose (0.6 M) and citrate phosphate buffer (0.02 M), pH 5.6 at 31°C. When the protoplasts were grown in the regeneration medium containing yeast extract (1.5%), 1 l of Mandel's salts, pH 5.6, and glucose (0.8 M), a high frequency of regeneration of the protoplast was obseved: 66% for *T. koningii* and 45% for *T. harzianum*. Two patterns of regeneration were observed. First, the hyphae arose directly from the regenerated protoplast mother cell. Second, a chain of bud cells developed from the protoplast and subsequently generating hyphae generally protruded from the terminal bud cells.

Key Words——protoplast formation; protoplast regeneration; *Trichoderma harzianum*; *Trichoderma koningii*.

Introduction

Members of the genus *Trichoderma* Persoon ex Fries are known to be effective biological control (biocontrol) agents for use against plant pathogenic fungi such as *Rhizoctonia solani* Kühn. The antagonists of *R. solani*, *T. koningii* Oudemans and *T. harzianum* Rifai were observed in previous work on the control of basal stem rot of chrysanthemum (Tschen et al., 1989).

Protoplast manipulation is an efficient tool for fungal biotechnology, having been used for intraspecific, interspecific and intergeneric hybridization and also for the improvement of industrial fungal strains (Ohnuki et al., 1982; Quigley et al., 1987). Preparation of protoplasts from *Trichoderma* was reported (Toyama et al., 1983; Stasz et al., 1988; Wang et al., 1988; Pe'er and Chet, 1991). However, these results were dependent upon various growth media, cell wall-lytic enzymes, osmotic stabilizers and physical conditions (Leong and Berka, 1991). The optimal conditions to produce the maximal yield of protoplasts from biocontrol fungal agents have not yet reported. We have investigated the optimal conditions for formation and regeneration of protoplasts from *T. koningii* and *T. harzianum*.

Materials and Methods

Microorganism *Trichoderma koningii* CHU 159 (T12) and *Trichoderma harzianum* CHU 157 (TVCN1) are antibiotic-producing antagonists to *Rhizoctonia solani*. This antagonistic effect was previously reported (Tschen et al., 1989).

Growth media The following media were used for growth of the fungi. Yeast extract agar (YA) (g/l): Yeast extract, 4; malt extract, 15; glucose, 4; agar, 20; pH 7.3; agar was omitted in the yeast extract broth (YB). Malt extract agar (MA) (g/l): Malt extract, 20; agar, 20. Mandel's salts (MS) (g/l): KH_2PO_4, 4; $(NH_4)_2SO_4$, 2.8; urea, 0.6; $CaCl_2 \cdot 2H_2O$, 0.6; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 5H_2O$, 0.0032; $ZnSO_4 \cdot 7H_2O$, 0.0028; $CoCl_2 \cdot 6H_2O$, 0.004; pH 5.6. Protoplast regeneration medium (PRM) (g/l): MS (or malt extract, 10), yeast extract, 15; glucose, 140 (0.8 M); agar, 20.

Osmotic stabilizer The osmotic stabilizer (OS) was prepared in citrate phosphate buffer (0.02 M), pH 5.6 containing sugar (glucose or sucrose, 0.4–0.8 M), sugar alcohol (mannitol or sorbitol) or inorganic salt (NaCl, $MgSO_4$). The buffer system containing mannitol (0.6 M) was used as a standard stabilizer for subsequent tests.

Cell wall-lytic enzyme solution The cell wall-lytic enzyme solution (LES) was prepared with the osmotic stabilizer and one of the following enzymes: (1) Novozym 234 (15 mg/ml, Sigma), (2) Driselase (20 mg/ml, Sigma), (3) cellulase "Onozuka" R-10 (20 mg/ml, Yakult Biochemical, Japan), (4) β-glucuronidase (0.06 mg/ml, Sigma), (5) Macerozym (5 mg/ml, Yakult Biochemical), (6) chitinase (2 mg/ml, Sigma).

Isolation of protoplasts A spore suspension of fungus (10^6 cell/ml) was grown in YB (100 ml) with shaking at 140 rpm and at 27°C for 20 h. The mycelia were collected on a sieve, washed with distilled water, then put on filter paper to absorb the remaining water. The mycelia were incubated in LES (5 ml) at 75 rpm and 31°C for 3–5 h. The mycelium suspension was passed through a sieve with pore size of 36 μm and the filtrate was centrifuged at $1000 \times g$ for 10 min to collect protoplasts.

The protoplasts were washed twice with OS containing mannitol (0.6 M), then collected in the stabilizer (30 ml). The number of protoplasts was estimated with a haemocytometer. Preparation of protoplasts was carried out under sterile conditions. Seven enzyme mixtures prepared from the LES (Table 2) were tested to determine the best enzyme for protoplast release.

To determine the optimum conditions for protoplast formation, various concentrations of Novozym (5, 10, 15, 20 mg/ml), culture ages of fungi (20, 30, 40 h) and concentrations of mycelia (20, 40, 60, 80, 100 mg/ml) were used. Protoplast formation was observed at various temperatures (25°, 31°, 37°C). The effect of pH on the production of protoplasts from *Trichoderma* was tested in the osmotic stabilizer containing mannitol (0.6 M) and the buffer system of citrate phosphate (0.02 M), with pH values in the range of 4.6-8.1.

Two kinds of osmotic stabilizer, organic (sucrose, mannitol, or sorbitol) and inorganic (magnesium sulfate, or sodium chloride), was investigated (Figs. 12, 13) to assess their optimal concentration for protoplast formation.

Regeneration of protoplasts Diluted protoplast suspensions (PS; 250 μl) were plated on the surface of protoplast regeneration medium (PRM). The PRM plates were incubated at 27°C for 2-3 days and the regeneration frequency (RF) was estimated according to the following formula (Ohnuki et al., 1982).

$$RF = \frac{\text{number of colonies on PRM}}{\text{number of protoplasts in PS}}$$

The regeneration process of protoplasts was observed both in broth and on agar media. (1) The protoplast (10^6/ml) suspension was mixed with PRM (20 ml), and the growth of mycelia from protoplasts was observed with a Zeiss Axiophotomicroscope. (2) The protoplast suspension (1 ml) was mixed with PRM (10 ml) containing agar (1%) at 45°C. The PRM agar was poured onto sterile glass slides ($15 \times 10 \times 0.15$ mm^3) to produce thin-layer agar (TLA) 0.15 mm thick (Kobayashi et al., 1985). The TLA was placed in a sterile moisture chamber at 27°C, and the growth of mycelia from protoplasts was observed microscopically.

The regeneration frequencies of protoplast preparations were tested in two series of protoplast regeneration media. (1) PRM1: Mandel's salts, 1.5% yeast extract and osmotic stabilizer. (2) PRM2: 1% malt extract, 1.5% yeast extract and osmotic stabilizer. Eight solutes were used as osmotic stabilizer in PRM (Table 3).

Results

Morphological characteristics of *Trichoderma* antagonists *Trichoderma koningii* CHU159 and *T. harzianum* CHU157 were isolated from Taiwan soils. The morphological characteristics of the strains are first described in this paper, as this is useful for comparing the morphology of the original and the regenerated strains. The results indicate that there were no morphological differences between the original and the regenerated strains. *T. koningii* and *T. harzianum* were grown on YA and MA at 27°C for five days. The morphology of fungi was easily distinguished by macroscopic and microscopic observations. The morphological characteristics of the fungi are shown in Table 1.

Table 1. Morphological characteristics of *T. koningii* and *T. harzianum*.

	T. koningii	*T. harzianum*
Hypha	colorless, septate, smooth	colorless, septate, smooth
Sporulation on YA	3 days	3 days
Sporulation on MA	>5 days	5 days
Color of spore	dark green	green
Kind of spore	phialospore, chlamydospore (terminal, intercalary)	phialospore, chlamydospore (terminal, intercalary)
Size of phialide (μm)	$7.5\text{-}16.0 \times 2.5\text{-}3.5$	$5.1\text{-}14.5 \times 1.5\text{-}3.3$
Size of phialospore (μm)	$3.0\text{-}4.8 \times 1.9\text{-}2.8$ spheroid	$2.3\text{-}3.3 \times 2.3\text{-}3.2$ spheriform, pyriform

Formation of protoplast from hyphae Lysis of the fungal wall was observed under the microscope following treatment of mycelia with Novozym 234 for 10 min. Protoplasts were initially released from terminal cells of hypha, where the cell wall first broke down. Protoplasts were released from intercalary cells when the cell wall was broken (Fig. 1, A). The protoplasts were the spherical in shape. During the early stages, after about 1 h of

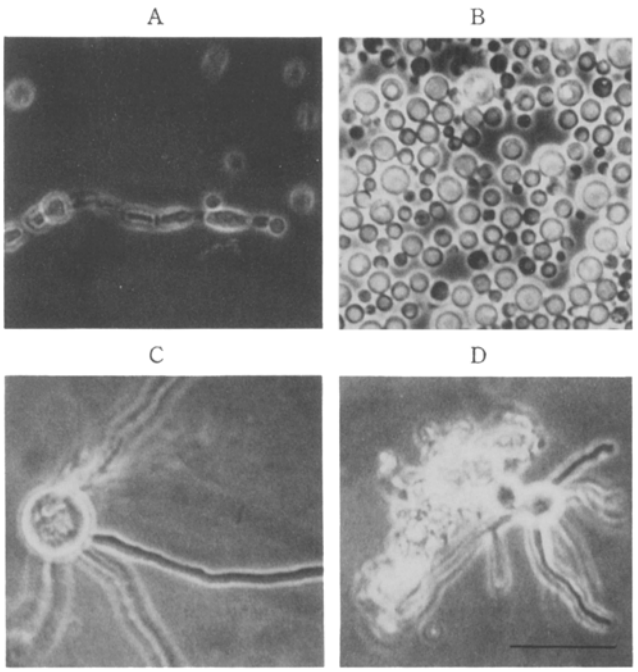

Fig. 1. Formation and regeneration of protoplasts from *Trichoderma*. A. Release of protoplasts from hypha of *T. koningii*; B. protoplasts of *T. koningii* isolated after 4 h of treatment with lytic enzyme; C. hyphae developed from a protoplast of *T. harzianum*; D. hyphase developed from a chain of bud cells of *T. harzianum*. Scale bar indicates 10 μm.

Table 2. Effect of lytic enzyme combinations on protoplast formation from *T. koningii* and *T. harzianum*.

Enzyme and formation of protoplast	Lysing solution[a]						
	1	2	3	4	5	6	7
Cellulase "Onozuka" R-10	+	+	−	+	+	−	−
Macerozym	+	+	−	+	+	−	−
Driselase	+	−	+	−	+	−	+
β-Glucuronidase	+	+	−	+	+	−	−
Chitinase	−	−	−	+	+	−	−
Novozym 234	−	−	−	−	−	+	+
Protoplasts/ml							
T. koningii	4.1×10^7	1.8×10^5	1.3×10^6	8.1×10^6	3.5×10^7	4.4×10^7	8.6×10^7
T. harzianum	4.3×10^7	3.4×10^5	1.8×10^6	4.0×10^6	4.2×10^7	3.1×10^7	6.6×10^7

[a] The solution contained citrate phosphate buffer (0.02 M) and mannitol (0.6 M) at pH 5.6. The age of mycelia was 20 h. The treatment temperature was 31°C for 3 h. The concentration of enzymes was cellulase, 20 mg/ml; Macerozym, 5 mg/ml; Driselase, 20 mg/ml; glucuronisidase, 0.06 mg/ml; chitinase, 2 mg/ml; and Novozym, 15 mg/ml.

treament with the cell wall-lytic enzyme, the diameter of the protoplasts was 2.2-5.1 μm, and they contained a few small vacuoles. Over a prolonged period of digestion (8 h) the volume of protoplasts increased. The diameter of protoplasts was in the range of 4.5-18 μm, and they contained many vacuoles (Fig. 1, B).

Factors affecting protoplast formation

(1) **Lytic enzyme** Novozym was found to be the best enzyme for protoplast release from *T. koningii* and *T. harzianum*, with yields of up to 3×10^7/ml. Drisease was the next best, with a yield of protoplasts twenty times less than Novozym. The optimal yield of protoplasts was obtained (in total over 6×10^7/ml) when a mixture of the two lytic enzymes was used (Table 2).

(2) **Concentration of Novozym 234** A concentrations of 15 mg/ml or 20 mg/ml of Novozym in the cell wall lytic solution containing was found to be the most effective for protoplast production from *Trichoderma koningii* and *T. harzianum*, respectively (Figs. 2, 3). Higher enzyme concentration (20 mg/ml) depressed protoplast yield, although this was still higher than with enzyme levels of 10 mg/ml and less. The solution containing 15 mg/ml of Novozym was subsequently used as the standard cell wall-lysing solution in tests to determine other factors affecting optimum protoplast formation.

(3) **Culture age** According to our observations, the age of fungal culture is an important factor in protoplast formation. Young mycelium produced more protoplasts than old mycelium. *Trichoderma koningii* (Fig. 4) or *T. harzianum* (Fig. 5) primarily produced protoplasts from 20-h mycelia. A smaller yield was produced from 30-h mycelia and the smallest yield was from 40-h mycelia.

(4) **Concentration of mycelium** Mycelia of *Trichoderma* strains were suspended at concentrations of 20-100 mg/ml in the lysing mixture containing Novozym 234 (15 mg/ml). The most effective concentrations of mycelia were 80 mg/ml for *T. koningii* (Fig. 6) and 60 mg/ml for *T. harzianum* (Fig. 7).

(5) **Temperature** The temperature used for the lytic digestion was important factor for the release of pro-

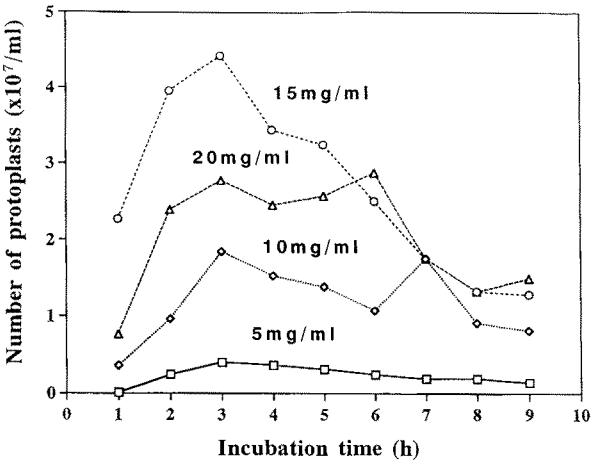

Fig. 2. Effect of the concentration of Novozym 234 on the release of protoplasts from *T. koningii*.

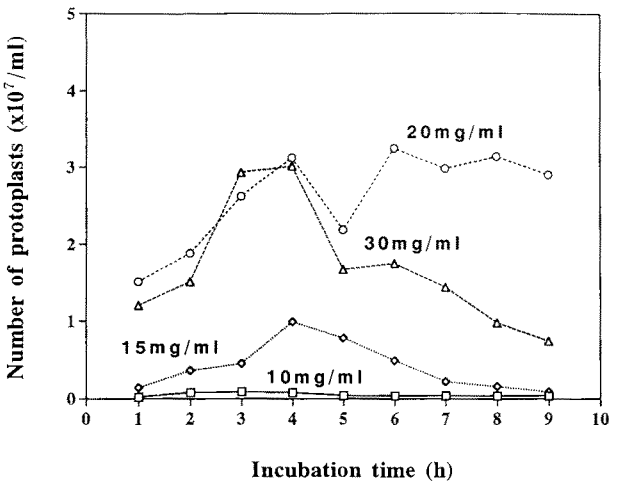

Fig. 3. Effect of the concentration of Novozym 234 on the release of protoplasts from *T. harzianum*.

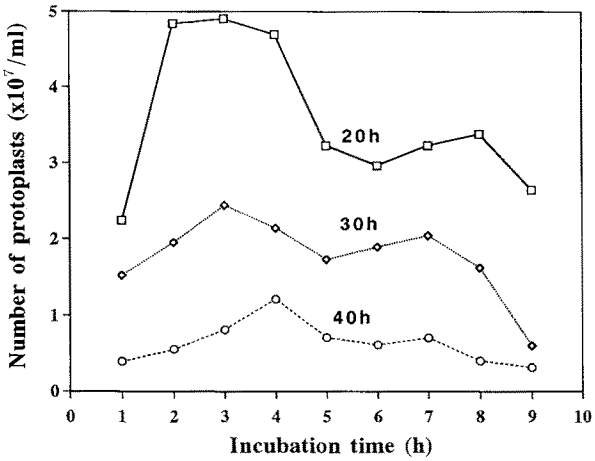

Fig. 4. Effect of culture age on the release of protoplasts from *T. koningii*.

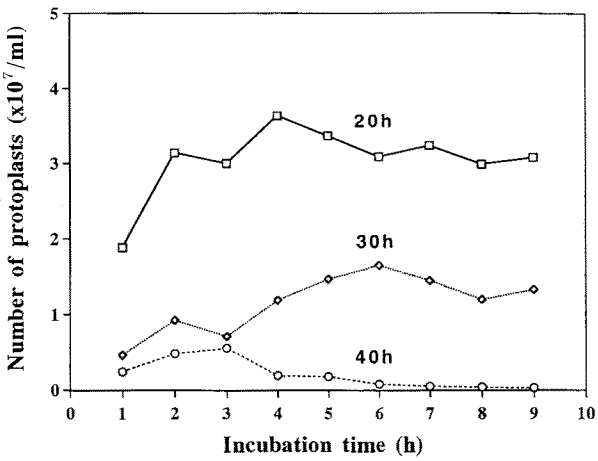

Fig. 5. Effect of culture age on the release of protoplasts from *T. harzianum*.

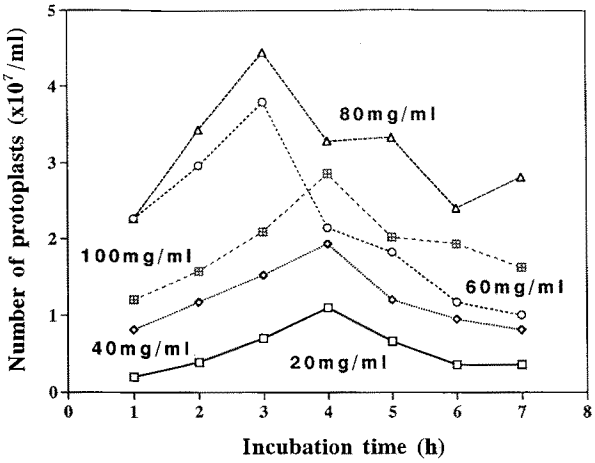

Fig. 6. Effect of mycelial concentration on the release of protoplasts from *T. koningii*.

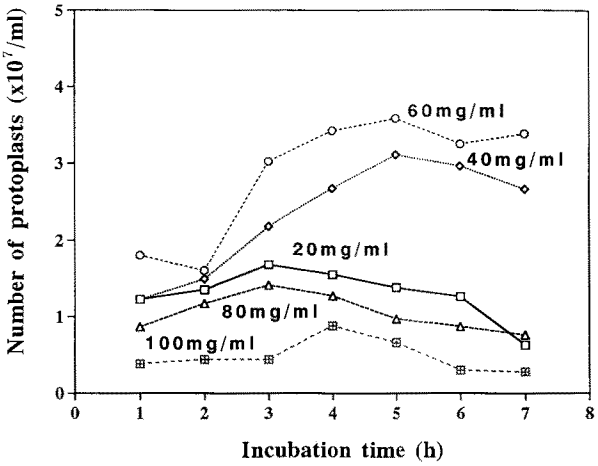

Fig. 7. Effect of mycelial concentration on the release of protoplasts from *T. harzianum*.

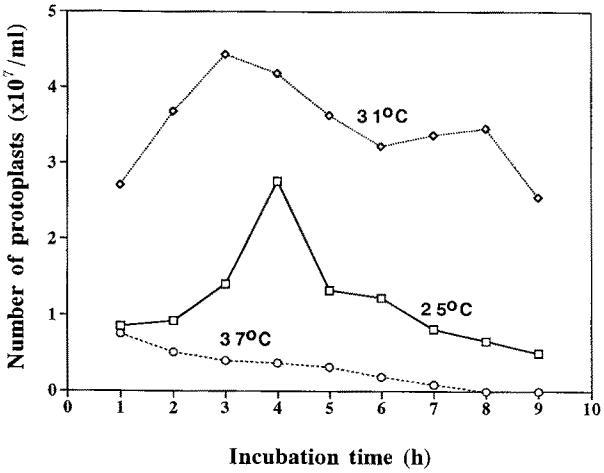

Fig. 8. Effect of temperature on the release of protoplasts from *T. koningii*.

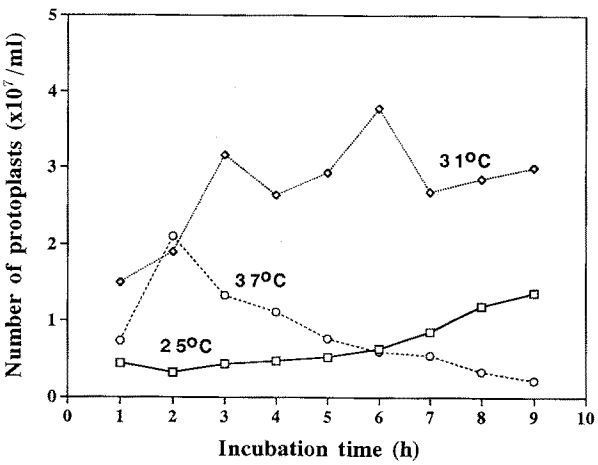

Fig. 9. Effect of temperature on the release of protoplasts from *T. harzianum*.

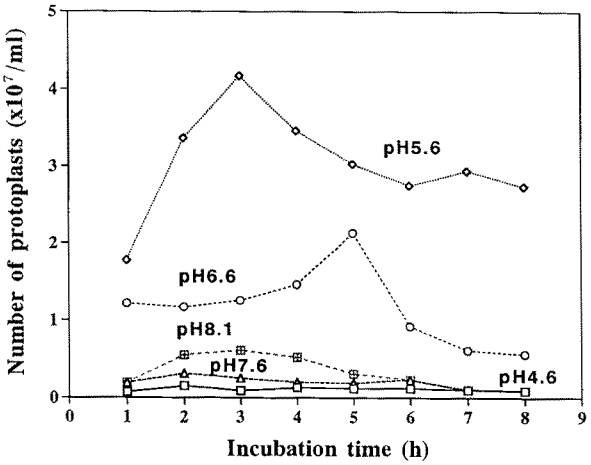

Fig. 10. Effect of pH value on the release of protoplasts from *T. koningii*.

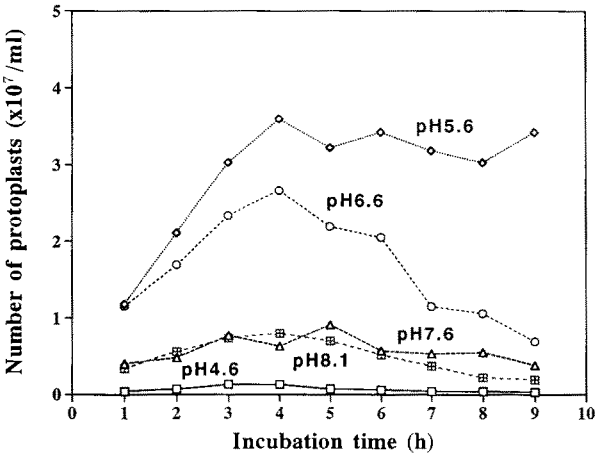

Fig. 11. Effect of pH value on the release of protoplasts from *T. harzianum*.

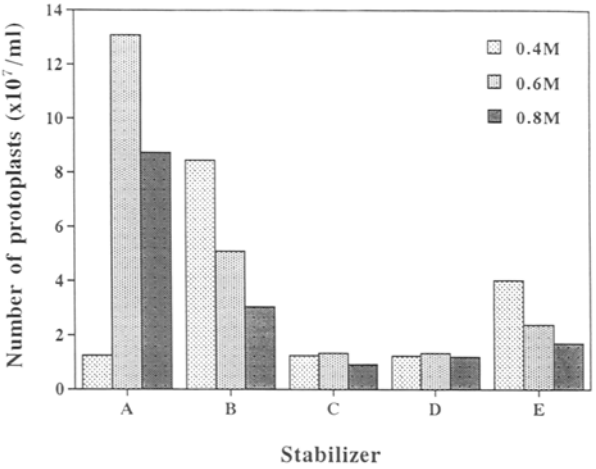

Fig. 12. Effect of type and concentration osmotic stabilizer on the release of protoplasts from *T. koningii*. A. Sucrose; B. mannitol; C. sorbitiol; D. magnesium sulfate; E. sodium chloride.

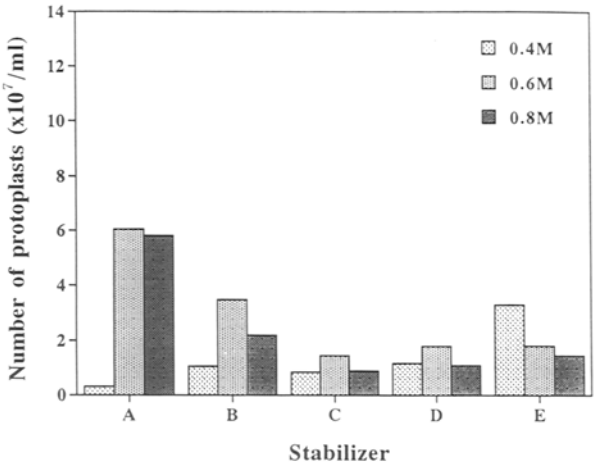

Fig. 13. Effect of type and concentration osmotic stabilizer on the release of protoplasts from *T. harzianum*. A. Sucrose; B. mannitol; C. sorbitiol; D. magnesium sulfate; E. sodium chloride.

toplasts from these fungi. The best yields were obtained from both species by incubation with Novozym 234 (15 mg/ml) at 31°C. *Trichoderma koningii* and *T. harzianum* are sensitive to higher temperatures, because productivity of protoplasts was minimum at 37°C (Figs. 8, 9), and the protoplasts shrank and assumed an irregular form.

(6) **pH** *Trichoderma koningii* (Fig. 10) and *T. harzianum* (Fig. 11) released protoplasts primarily at pH 5.6. When protoplasts were prepared at pH 4.6, lysis of protoplasts was observed two hours after release.

(7) **Osmotic stabilizer** Varying amounts of protoplasts were released in various osmotic stabilizers. The optimal concentration of osmotic stabilizer was 0.6 M for sucrose, mannitol, sorbitol and magnesium sulfate, but 0.4 M for sodium chloride. The most suitable stabilizer was sucrose (0.6 M), with protoplast yields of 1.2×10^8 /ml for *T. koningii* (Fig. 12) and 6×10^7/ml for *T. harzianum* (Fig. 13). The next most useful stabilizers were sodium chloride (0.4 M) and mannitol (0.6 M), with which both fungi produced yield of $3\text{-}4 \times 10^7$ protoplasts/ml. Protoplast yields produced in sorbitol (0.6 M) and magnesium sulfate were less 2×10^7/ml. The effect of the stabilizers ranged from three to six hours after incubation. The protoplasts started to lyse and to develop vacuoles when they were exposed to osmotic stabilizers for eight hours.

(8) **Incubation time in LES** Incubation time seems to be another important factor in improving production of protoplasts. In general, the optimal incubation time was 3 h for *T. koningii* and 4 h for *T. harzianum*.

Regeneration of protoplasts The Mandel's salt-yeast medium (PRM1) was efficient for protoplast regeneration in both *T. koningii* and *T. harzianum* (Table 3). The medi-

Table 3. Regeneration of protoplasts of *Trichoderma* species in media containing different osmotic stabilizers.

Regeneration medium	Regeneration frequency (%)	
	T. koningii	*T. harzianum*
Mandel's salt-yeast medium[a] with stabilizer:		
0.6 M glucose	57.2±7.5[c]	32.4±6.8
0.8 M glucose	66.0±10.2	44.6±9.4
0.6 M sucrose	43.2±6.8	20.5±7.1
0.8 M sucrose	42.8±7.2	26.3±4.7
0.6 M mannitol	32.4±4.6	17.5±5.6
0.8 M mannitol	33.7±2.9	20.8±3.4
0.4 M NaCl	26.9±13.0	26.2±4.9
0.6 M NaCl	31.5±7.7	28.7±3.5
blank	0.0	0.0
Malt-yeast medium[b] with stabilizer:		
0.6 M glucose	34.1±4.9	21.2±2.7
0.8 M glucose	38.8±8.5	24.0±3.4
0.6 M sucrose	24.8±7.5	12.8±4.6
0.8 M sucrose	30.2±9.6	14.1±5.8
0.6 M mannitol	23.6±2.4	16.2±1.4
0.8 M mannitol	24.3±1.2	15.3±2.4
0.4 M NaCl	22.2±5.1	20.9±2.6
0.6 M NaCl	28.2±4.6	22.4±4.0
blank	0.0	0.0

[a] Medium contained Mandel's salts and yeast extract (1.5%).
[b] Medium contained malt extract (1.0%) and yeast extract (1.5%).
[c] Means with standard deviations.

um containing glucose as stabilizer proved most effective. The highest average regeneration frequency of protoplasts in PRM containing glucose (0.8 M) was 66% for *T. koningii* and 44.6% for *T. harzianum.* The highest regeneration frequency in the malt-yeast medium (PRM2) was only 38.8% for *T. koningii* and 24% for *T. harzianum.* The regeneration frequency of *T. koningii* always exceeded that of *T. harzianum.*

Observation on protoplast regeneration The regeneration of purified protoplasts was observed on TLA. The vacuoles in the protoplasts shrank during the regeneration and finally disappeared. New hyphae started to develop from protoplasts after 6 h and were present in abundance on the TLA after 10 h. The development of hyphae in the liquid protoplast regeneration media was observed after 10 h of incubation. However, the development of hyphae demonstrated similar pictures both on the TLA and in the liquid media. Regeneration of protoplasts was observed in two ways: (1) the hyphae arose directly from the regenerated protoplast mother cells (Fig. 1, C); and (2) the hyphae arose from the terminal buds or bud cells in the chain that was produced originally from protoplasts (Fig. 1, D).

Discussion

The productivity of protoplasts from *T. koningii* and their regeneration frequency were characteristically greater than those of *T. harzianum.* This may depend upon various properties of the cell wall. For instance, the structure and composition of fungal cell wall vary from species to species. The preparation and regeneration of protoplasts was affected by the fungal cells and by the treatment conditions, i.e., ages of mycelia, cell wall-lytic enzymes, concentrations of mycelium mass, osmotic stabilizers, temperature and pH (Peberdy, 1987). The optimization of production and regeneration of protoplasts from *Trichoderma* species is useful in biotechnological investigation of these filamentous fungi, e.g., transformation (Leong and Berka, 1991).

Of the six cell wall-lytic enzymes employed, Novozym exhibited the best lysing effect against the two species investigated. This enzyme complex has been used for preparation of protoplasts from many different fungi including Zygomycotina, Ascomycotina, Besidiomycotina and Deuteromycotina (Kitamoto et al., 1987).

The age of mycelium is considered to be an important factor in preparing protoplasts. Protoplasts were produced abundantly when prepared from the young mycelia of *Trichoderma*, but yield of protoplast decreased markedly when mycelia older than 20 h after transplantation were used, probably because the old mycelia had become more resistant to the lytic enzymes (Peberdy, 1987). We recently observed that the age of mycelium was a critical factor in preparation of protoplasts from the plant pathogenic fungus *Rhizoctonia solani* (unpublished data).

The osmotic stabilizer is an important component of the medium in maintaining a balanced osmotic potential for the isolated protoplasts and also in protection of the protoplast membrane from osmotic shock (Lynch et al., 1985). Some isolated protoplasts of *Trichoderma* became deformed after 8-9 h incubation, despite the large amount of protoplasts obtained in the osmotic stabilizer containing sucrose (0.6 M). A practical osmotic stabilizer containing mannitol (0.6 M) is suggested to be effective for long-term usage and also to maintain the quality of protoplasts.

Temperature and pH are biological environmental factors that affect the reactions of the lytic enzymes and the effects of the osmotic stabilizers (Pe'er and Chet, 1991). The high production of protoplasts from *Trichoderma* species at 31°C and pH 5.6 indicates that reactions of Novozym are suitable at this temperature and pH. The protoplast membrane system might become damaged at a temperature higher than 37°C or greater concentration of hydrogen ion than at pH 4.6. These conditions are unfavorable for protoplasts and lead to shrinking or decomposition of protoplasts. The production of protoplasts was severely diminished.

According our results, the best conditions to produce the maximal yield of the protoplasts from *Trichoderma* are as follows: 15 mg/ml of Novozym, 20-h mycelia, 80 mg/ml of mycelium, 0.6 M sucrose as stabilizer; incu-

bation of the lytic mixture at 31°C and pH 5.6 for 3 h.

The protoplasts were cultured on solid and liquid growth media for regeneration of hyphae. The solid medium demonstrated greater efficiency than the liquid meidum, possibly owing to better aeration, as some protoplasts might lose the ability to regenerate in anaerobic conditions. Some isolated protoplasts were unable to regenerate when their membranes were destroyed by lytic enzymes (Quigley et al., 1987) or when they included no nuclei (Akamatsu et al., 1983). The protoplast regeneration medium containing glucose was useful in functioning as an osmotic stabilizer and in providing the essential energy source and the monomers for synthesizing glucans and other fungal wall components (Moore and Peberdy, 1976).

The protoplasts isolated in this work are expected to be a suitable in quality and quantity as a source for molecularbiologial studies. These methods are also useful to other filamentous fungi.

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