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Protoplast Formation and Regeneration from Mycelia of *Phytophthora capsici*

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*Phytophthora capsici*의 菌絲體로부터 原形質體 形成과 再生

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ABSTRACT: Factors responsible for protoplast formation and regeneration of *Phytophthora capsici* were examined. Protoplasts were successfully liberated from the mycelial culture by digestion for 6-9 hrs with Novozym 234 in 0.35 M $CaCl_2$ (pH 5.7) as osmotic stabilizer. Young rapidly-growing mycelium (24 hrs old) showed highest protoplast yields. High concentrations of Novozym 234 were effective in releasing protoplasts from the mycelium. The combination of 0.4 M mannitol and 0.1 M $CaCl_2$ was optimal osmotic stabilizers for protoplast regeneration. The synthetic Henninger media containing all nutritional elements gave the best regeneration rate. The protoplast regeneration was greatly inhibited in the media which were not supplement with amino acids or β-sitosterol. Certain amino acids such as L-aspartic acid and L-glutamic acid remarkably enhanced protoplast regeneration. However, the addition of microelements did not affect protoplast regeneration.

KEYWORDS: *Phytophthora capsici*, protoplast formation, protoplast regeneration.

Protoplasts are widely being used for the physiological, biochemical, and genetic research in microorganisms (Harris, 1982; Peberdy and Ferenczy, 1985). The formation and regeneration of protoplasts of yeast has earlier been studied (Duel *et al.*, 1964; Bacon *et al.*, 1969; Novaes-Ledieu and Garcia-Mendoza, 1970). The methods of protoplast formation and regeneration in plant pathogenic fungi have been developed to investigate the mechanisms of pathogenicity or genetics of them (Tanaka *et al.*, 1981; Harris, 1982; Ishizacki *et al.*, 1983; Peberdy and Hocart, 1987; Lynch *et al.*, 1989). Recently, formation and regeneration of protoplasts have been extensively studied in *Phytophthora* spp. (Jahnke *et al.*, 1987; Layton and Kuhn 1988a, b; Nam, 1988; Campbell *et al.*, 1989; Lucas *et al.*, 1990) after the first observation of protoplast liberation of the fungi by Bartnicki-Garcia and Lippman (1966).

Protoplast formation is affected by culture age, osmotic stabilizer, and lytic enzymes (Peberdy, 1979; Peberdy and Ferenczy, 1985). Protoplasts are efficiently produced from young rapidly-growing mycelium in *P. parasitica* (Jahnke *et al.*, 1987), *P. megasperma* f. sp. *glycinea* (Layton and Kuhn, 1988a, b) and *P. capsici* (Nam, 1988; Lucas *et al.*, 1990), but not in *P. parasitica* and *P. cinnamomi* (Bartnicki-Garcia and Lippman, 1966). Protoplasts can be obtained from sporangia and zoospores of *Phytophthora*. Since sporangia of *P. infestans* are deciduous, therefore, sporangia and encysted zoospores are used as a starting material for protoplast formation (Campbell *et al.*, 1989). The efficiency of formation and regeneration of protoplast from sporangia and encysted zoospores is higher than those from mycelium. In the case of *P. capsici*, however, it is difficult to release protoplasts from the sporangia, because sporangia of the

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fungus are nondeciduous. Inorganic compound such as $CaCl_2$, KCl, and $MgSO_4$, and the organic substances such as sorbitol and mannitol have widely been used as osmotic stabilizers with a mixture or alone for protoplast formation of *Phytophthora* (Bartnicki-Garcia and Lippman, 1966; Jahnke *et al.*, 1987; Layton and Kuhn, 1988a, b; Nam, 1988; Campbell *et al.*, 1989; Lucas *et al.*, 1990). Extracellular enzyme preparation from *Streptomyces* sp. was first used as a lytic enzyme for protoplast formation of *P. parasitica* and *P. cinnamomi* (Bartnicki-Garcia and Lippman, 1966). Novozym 234, Driselase or Cellulase have recently been used in *Phytophthora* (Layton and Kuhn, 1988a; Lucas *et al.*, 1990).

The efficient regeneration of protoplasts into mycelial cells is achieved by optimal regeneration media and osmotic stabilizer. Natural media such as potato dextrose agar, V-8 juice medium, and pea extract broth containing osmotic stabilizers were well suited for protoplast regeneration of *Phytophthora* (Campbell *et al.*, 1989). The osmotic stabilizer for protoplst regeneration differed from that for protoplast formation in *P. parasitica* (Jahnke *et al.*, 1987). The optimal conditions for formation and regeneration of protoplasts are different between *Phytophthora* species.

The objective of this study was to examine which factors and environmental conditions are most essential for protoplast formation and regeneration from mycelia of *P. capsici*.

Materials and Methods

Fungal isolate and culture media: The isolate 87L19 of *Phytophthora capsici* was used in this study. All cultures were maintained on V-8 juice agar at room temperature.

Oatmeal medium, V-8 juice medium, pea broth extract medium, and Henninger medium were used in this study. The oatmeal media were prepared by boiling 30g of oatmeal power in 1 *l* of distilled water and filtering through two sheets of cheesecloth. The oatmeal broth was added with 20g of agar and adjusted to 1 *l* with distilled water. The pea broth extract media were prepared by boiling 200g of frozen pea in 1 *l* of distilled water for 45 min and filtering through two sheets of cheesecloth. After centrifugation at 1,200g for 20 min, the clarified pea broth extract was supplemented with 5g of glucose and then adjusted to 1 *l* with distilled water. Two kinds of V-8 juice media were used: one was clarified with $CaCO_3$ and the other was unclarified. The V-8 juice was centrifuged at 1,200g for 20 min to get a clarified supernatent. To determine which of nutritional elements may be essential for the regeneration of protoplasts, the synthetic Henninger media wre prepared by varying the nutritional composition in the media. The compositions of Henninger media used in this study were described in Table 1.

Production of protoplasts: The 10 m*l* of zoospore suspension (4×10^5/m*l*) was poured to 90 m*l* of sterile pea broth extract in a 250 m*l* fask. The flasks were incubated at 27℃ on a reciprocal shaker at 60 rpm for 20-120 hr. The mycelial cultures grown for 36 hr were used as a standard inoculum for protoplast production. Mycelia were harvested by suction-filtering on a Büchner funnel and washed with sterile distilled water.

Osmotica I and II were used as a osmotic stabilizer. Osmoticum I (0.35 M $CaCl_2$) was used to produce protoplasts, and osmoticum II (0.1 M $CaCl_2$, 0.4 M mannitol) was used to wash enzyme and to dilute protoplasts. Both osmotica were adjusted to pH 6.2 and the pH dropped to 5.7 after autoclaving.

One gram of wet fresh mycelium was suspended into 20 m*l* of osmoticum I containing 3 mg/m*l* Novozym 234 (Sigma) in a 100 m*l* flask and the flask was incubated on a reciprocal shaker at 26℃, 100 rpm for 5 hr.

Protoplasts were separated from mycelial fragments in the enzyme solutions by filtering through 100 μm and 40 μm nylon meshes. The filtrate was centrifuged at 300 g for 15 min. The supernatant was decanted and the pellet was washed at least twice with 5 m*l* of osmoticum II by centrifugation at 300 g for 15 min. The intact protoplasts were purified by the method of Hashiba and Yamada (1982). The pellet was suspended in 2 m*l* of osmoticum II and layered onto 4 m*l* of HEPES solution

Table 1. Various nutrient compositions of synthetic Henninger media used for the study of protoplast regeneration of *Phytophthora capsici.*

Element	Concentration	Nutrient composition number					
		1	2	3	4	5	6
C-source							
sucrose	15.0 (g/*l*)	+[a]	+	+	+	+	+
N-source							
$NaNO_3$	400.0 (mg/*l*)	+	+	+	+	+	+
$(NH_4)_2SO_4$	100.0 (mg/*l*)	+	+	+	+	+	+
Macroelements							
K_2HPO_4	300.0 (mg/*l*)	+	+	+	+	+	+
KH_2PO_4	400.0 (mg/*l*)	+	+	+	+	+	+
$CaCl_2 \cdot 2H_2O$	100.0 (mg/*l*)	+	+	+	+	+	+
$MgSO_4 \cdot 7H_2O$	500.0 (mg/*l*)	+	+	+	+	+	+
Microelements							
$FeSO_4 \cdot 7H_2O$	10.0 (mg/*l*)	+	+	+	−	−	+
$ZnSO_4 \cdot 7H_2O$	1.8 (mg/*l*)	+	+	+	−	−	+
$CuSO_4 \cdot 5H_2O$	0.4 (mg/*l*)	+	+	+	−	−	+
$MnSO_4 \cdot H_2O$	0.3 (mg/*l*)	+	+	+	−	−	+
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	0.3 (mg/*l*)	+	+	+	−	−	+
Amino acid							
DL-alanine	100.0 (mg/*l*)	+	−	−	+	−	+
L-arginine	200.0 (mg/*l*)	+	−	−	+	−	+
L-aspartic acid	400.0 (mg/*l*)	+	+	−	+	−	+
Cystein	200.0 (mg/*l*)	+	−	−	+	−	+
L-glutamic acid	100.0 (mg/*l*)	+	+	−	+	−	+
Glycine	200.0 (mg/*l*)	+	−	−	+	−	+
Leucine	100.0 (mg/*l*)	+	−	−	+	−	+
Vitamine							
Thiamine	1.0 (mg/*l*)	+	+	+	+	+	+
Organic acid							
Succinic acid	200 (mg/*l*)	+	+	+	+	+	+
β-sitosterol	[illegible].0 (mg/*l*)	+	+	+	+	+	−

[a] + : presence of the corresponding element, − : absence of the corresponding element.

(0.6 M sucrose, 1 mM CaCl, 5 mM HEPES-KOH, pH 7.0). The solution was centrifuged with a swing bucket rotor at 100 g for 10 min. The intact protoplasts located at the interface of the two phase system were removed with a sterile pasteur pipette, and resuspended in 1 m*l* of osmoticum II. The protoplasts in osmoticum II were counted with a hemacytometer.

Regeneration of protoplasts: Various osmotica, consisting of different concentrations of $CaCl_2$ and mannitol, and the synthetic nutrient media were tested for regeneration of protoplasts. The regeneration media were adjusted to pH 6.2 before sterilized. All of the synthetic Henninger media were sterilized at 105℃ for 30 min. Osmoticum II (0.1 M $CaCl_2$, 0.4 M mannitol) was used as an osmotic stabilizer for regeneration of protoplasts on the Henninger media.

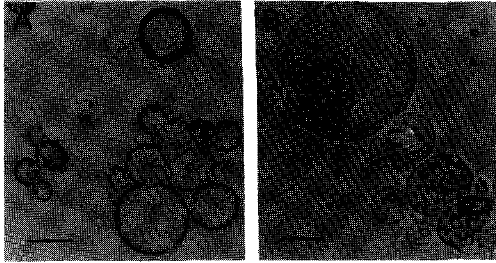

Fig. 1. Protoplasts produced from the mycelium of *Phytophthora capsici* treated for 5 hr at 26℃ with 20 mg·ml^{-1} Novozym 234 in 0.35 M $CaCl_2$. (A) Phase contrast micrograph of protoplasts in different shapes. (B) Micrograph of intercalary protoplasts emerging in a chain from the lysed mycelium.
Bars represent 10 μm.

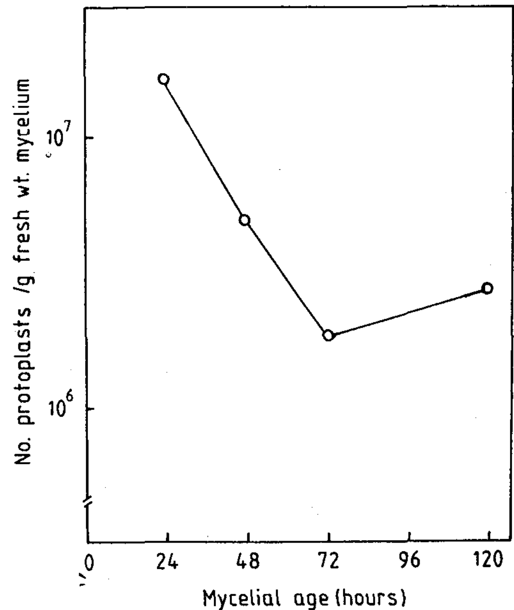

Fig. 2. Protoplast formation from the mycelium of *Phytophthora capsici* isolate 87L19 of different ages incubated for 5 hr at 0.5 mg·ml^{-1} Novozym 234 in 0.35 M $CaCl_2$.

The 500 μl of a series of diluted suspensions of protoplasts was gently spread on various regeneration media. The inoculated plates were incubated at 27℃ for 3-4 days. During the incubation, colonies were counted everyday. The rate of protoplast regeneration was expressed as % regeneration=(No. colonies/No. protoplasts)×100.

Results

Protoplast formation: After the treatment with Novozym 234 in 0.35 M $CaCl_2$ as a osmotic stabilizer for 30-60 min, the mycelia of *P. capsici* became loosed and swollen. The protoplasts began then to protrude from the burst tips of hyphae. After incubation for 2 hr, the subapical region of hyphae became lysed and protoplasts also were released from the lysed cell wall. The liberated protoplasts became swollen and larger during the incubation. Normal protoplasts were spherical and uniform in appearance, but irregularly burged bodies were also observed (Fig. 1A). Protoplast sizes ranged from 10 to 40 μm in diameter. Several intercalary protoplasts of different sizes sometimes remained connected in a chain, possibly originating from one hypha (Fig. 1B).

Protoplast yield was affected by the age of the mycelium of *P. capsici* (Fig. 2). Increased age of the mycelium reduced the liberation of protoplasts

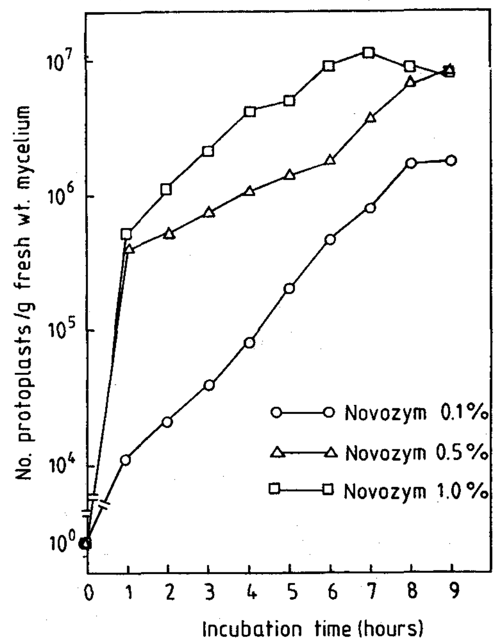

Fig. 3. Protoplast formation from the 36 hr-old mycelium of *Phytophthora capsici* isolate 87L19 incubated for different times at different concentrations of Novozym 234 in 0.35 M $CaCl_2$.

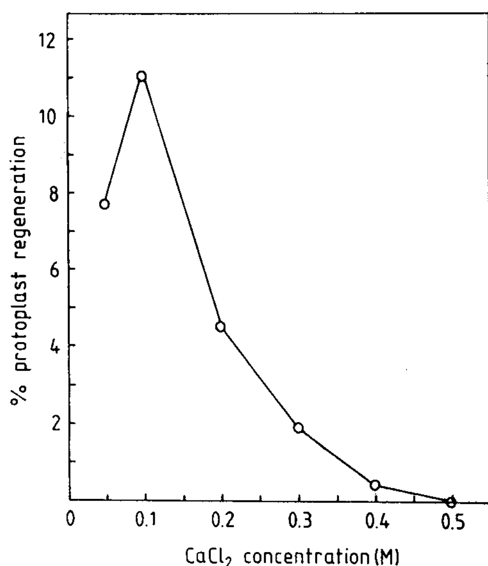

Fig. 4. Protoplast regeneration of *Phytophthora capsici* isolate 87L19 at different concentrations of $CaCl_2$ and 0.4 M mannitol in V-8 agar after incubation for 3 days.

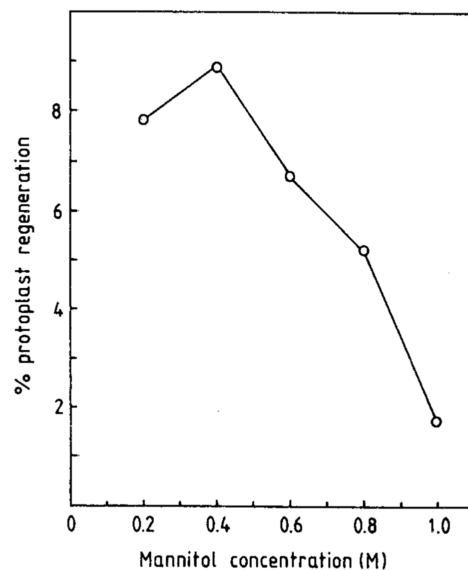

Fig. 5. Protoplast regeneration of *Phytophthora capsici* isolate 87L19 at different concentrations of mannitol and 0.1 M $CaCl_2$ in V-8 agar after incubation for 3 days.

from the mycelium. Young mycelium (24 hr old) gave highest protoplast yields. The concentration of Novozym 234 used for digestion also exerted influence on protoplast formation (Fig. 3). With increasing of the concentration of Novozym 234, protoplasts were rapidly released from the mycelium during the incubation in the enzyme solution. The high yields of protoplasts occurred within 6-9 hr by treating the mycelium with Novozym 234. Most of the protoplasts obtained by the digestion of mycelium with Novozym 234 beyond 5 hr were readily lysed after centrifugation to pellet the protoplasts. However, the addition of Novozym 234 after initial digestion with Driselase prevented the lysis of the protoplasts.

Protoplast regeneration: Protoplasts regenerated into new mycelium and colonies when spread on a medium with suitable osmotic stabilizers and adequate nutrients. Mixtures of the two osmotica $CaCl_2$ and mannitol were examined for regeneration of protoplasts. At a constant concentration of 0.4 M mannitol, increasing amounts of $CaCl_2$ above 0.1 M decreased the regeneration of protoplasts (Fig. 4). The highest concentration of 0.5 M $CaCl_2$ did not regenerate protoplasts into new mycelium. At a constant concentration of 0.1 M $CaCl_2$, increasing amounts of mannitol above 0.4 M also decreased regeneration rate (Fig. 5). The regeneration rate of protoplasts was high when 0.05-0.2 M $CaCl_2$ were mixed with 0.2-0.6 M mannitol in the regeneration media. The optimal concentrations of $CaCl_2$ and mannitol for osmotic stabilizers were 0.1 M $CaCl_2$ and 0.4 M mannitol.

Protoplast regeneration was greatly dependent on the nutrient compositions in the regeneration media (Fig. 6). The synthetic Henninger media containing all nutritional elements gave the best regeneration rate among all nutrient media tested. The regeneration of protoplasts were greatly inhibited in the media which were not supplemented with amino acids or β-sitosterol. The two amino acids L-aspartic acid and L-glutamic acid were more essential for protoplast regeneration than the other amino acids added. However, the addition of microelements such as $FeSO_4$, $ZnSO_4$, $CuSO_4$, and $MnSO_4$ did not affect protoplast regeneration.

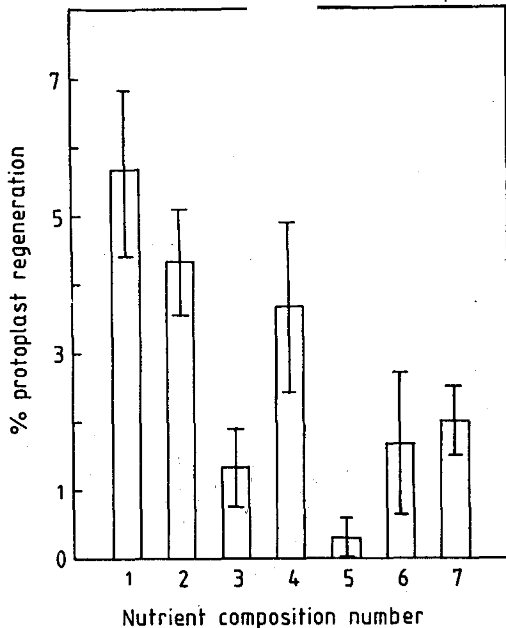

Fig. 6. Protoplast regeneration of *Phytophthora capsici* isolate 87L19 at different nutrient compositions of modified Henninger media (no. 1-6) and V-8 juice agar (no. 7). For nutrient compositions of Henninger media see Table 1.

Discussion

Protoplast yields are dependent on the age of the mycelium, lytic enzymes, and osmotic stabilizers (Peberdy, 1979). Protoplast yields of most filamentous fungi are highest during the exponential phase of growth, declining rapidly when stationary conditions are reached. This phenomenon was also consistent with *P. capsici*. Highest yields were obtained from the cultures incubated for 24-36 hr. Increasing the age of mycelium above 36 hr decreased protoplast yield.

The cell walls of *Phytophthora* are composed mainly of β-glucans (Bartnicki-Garcia and Lippman, 1966). Novozym 234 has been successfully used as a lytic enzyme to produce protoplasts from *P. parasitica* and *P. infestans* (Jahnke *et al.*, 1987; Campbell *et al.*, 1989). High concentrations of Novozym 234 were effective in releasing protoplasts from the mycelia of *P. capsici* (Fig. 3). In particular, Novozym 234 reduced the viability of protoplasts obtained by the digestion of mycelium beyond 5 hr, possibly as a result of the lysis of protoplast membranes by a significant amount of proteases (Lucas *et al.*, 1990). Recently, Layton and Kuhn (1988) obtained high yields of viable protoplasts by digesting rapidly growing mycelium with Driselase (a crude preparation of laminarinase, xylase, and cellulase). The 0.35 M $CaCl_2$ (pH 5.7), as an osmotic stabilizer, was well suited for protoplast formation of *P. capsici*, as previously suggested by Jahnke *et al.* (1987) with the study of *P. parasitica* spheroplasts.

It has been reported that intact protoplasts are purified by floating on the solutions of sucrose or sorbitol (Hashiba and Yamada, 1982; Kirimura, 1986; Huang *et al.*, 1990). In the present study, protoplasts could be seperated from mycelial fragment and cell debris using was HEPES-solution (0.6 M sucrose, 1 mM $CaCl_2$, 5 mM HEPES-KOH, pH 7.0). When protoplast suspension overlaid onto the HEPES-solution was centrifuged, most of mycelial fragments and broken protoplasts were sedimented into the lower phase and the purified protoplasts were at the interface of gradient. In contrast, the omission of the treatment with the HEPES-solution resulted in relatively numerous mycelial fragments and broken protoplasts in a protoplast suspension. Therefore, the choice of the treatment with HEPES-solution will depend on the degree of purity of protoplasts required for the experiments.

Viable protoplasts have the potential to synthesize new cell walls and grow to a mycelial state (Peberdy, 1979). The addition of osmotica such as $CaCl_2$ and mannitol in the regeneration media greatly affected the survival of protoplasts of *P. capsici* on the agar surface. The combination of 0.4 M mannitol and 0.1 M $CaCl_2$ was evaluated as optimal osmotic stabilizer for protoplast regeneration. Based on the observation of protoplast regeneration of *P. parasitica*, Jahnke *et al.* (1987) suggested a physical effect of osmotic stabilization provided by $CaCl_2$ and mannitol and a physiological effect caused by $CaCl_2$ on the cell membrane.

The nutritional elements in the media appear to be necessary for protoplasts to regenerate cell

walls. The protoplasts of *P. capsici* regenerated best in the synthetic Henninger medium containing all nutritional elements, although the colonies on this medium grew in an abnormal shape with the severe irregular margin and scattered aerial mycelia. Out of the nutrients, certain amino acids such as L-aspartic acid and L-glutamic acid enhanced protoplast regeneration, indicating that these amino acids may be some of the building blocks essential for the *de novo* synthesis of cell walls. Our findings that the mycelial growth of *P. capsici* on the natural V-8 juice media was faster than the synthetic Henninger media (no data presented) but the protoplast regeneration was better in the Henninger media may well suggest that the nutritional elements are different for mycelial growth and cell wall synthesis of *P. capsici*.

摘 要

*Phytophthora capsici*에서 원형질체를 형성, 재생시키는데 관여하는 요인에 대해 조사연구하였다. 삼투압조절제로서 0.35 M $CaCl_2$가 첨가된 Novozym 234를 6-9시간 처리하면 균사체에서 원형질체가 양호하게 나출되었다. 24시간 배양한 어린 균사체에서 가장 많이 원형질체를 나출시킬 수 있었으며, 또한 Novozym 234의 농도가 진할수록 효과적으로 원형질체가 나출되었다. 원형질체를 재생시키는데는 0.4 M mannitol과 0.1 M $CaCl_2$를 혼합한 것이 삼투압조절제이었다. 원형질체의 재생률은 모든 영양소가 첨가된 Henninger 합성배지에서 가장 높았다. 아미노산이나 β-sitosterol은 원형질체의 재생에 영향을 미쳐 두 영양소가 빠지면 원형질체의 재생이 억제되었다. 특히 아미노산 중 L-aspartic acid와 L-glutamic acid는 원형질체의 재생을 촉진시켰다. 그러나, 미량원소는 원형질체의 재생에 영향을 주지 않았다.

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References

Bacon, J. S. D., Jones, D. and Ottoleghi, P. 1969. Cell wall residues in yeast protoplast preparation. *J. Bacteriol.* **99**: 885-887.

Bartnicki-Garcia, S. and Lippman, E. 1966. Liberation of protoplasts from the mycelium of *Phytophthora. J. Gen. Microbiol.* **42**: 411-416.

Campbell, A. M., Moon, R. P., Duncan, J. M., Gurr, S-J. and Kinghorn, J. R. 1989. Protoplast formation and regeneration from sporangia and encysted zoospores of *Phytophthora infestans. Physiol. Mol. Plant Pathol.* **34**: 299-307.

Duell, E. A., Inoue, S. and Utter, M. F. 1964. Isolation and properties of intact mitochondria from spheroplasts of yeast. *J. Bacteriol.* **88**: 1762-1773.

Harris, G. M. 1982. Protoplast from *Gibberella fujikuroi. Phytopathology* **72**: 1403-1407.

Hashiba, T. and Yamada, M. 1982. Formation and purification of protoplasts from *Rhizoctonia solani. Phytopathology.* **72**: 849-853.

Huang, D., Staples, R. C., Bushnell, W. R. and Maclean, D. J. 1990. Preparation and regeneration of protoplasts from axenic mycelia derived from the wheat stem rust fungus. *Phytopathology* **80**: 81-84.

Ishizaki, H., Yajima, A., Kohno, M. and Kunoh, H. 1983. Effect of isoprothiolane on *Phricularia oryzae* Cav. (1) Cell wall regeneration of protoplasts. *Ann. Phytopath. Soc. Japan* **49**: 471-480.

Jahnke, K. D., Leipoldt, G. and Prell, H. H. 1987. Studies on preparation and viability of *Phytophthora parasitica* spheroplast. *Trans. Br. Mycol. Soc.* **89**: 213-220.

Kirimura, K., Yaguchi, T. and Usami, S. 1986. Intraspecific protoplast fusion of citric acid-producing strains of *Aspergillus niger. J. Ferment. Technol.* **64**: 473-479.

Layton, A. C. and Kuhn, D. N. 1988a. Heterokaryon formation by protoplast fusion of drug-resistant mutants in *Phytophthora megasperma* f. sp. *glycinea. Exp. Mycol.* **12**: 180-194.

Layton, A. C. and Kuhn, D. N. 1988b. The virulence of interracial heterokaryons of *Phytophthora megasperma* f. sp. *glycinea. Phytopathology* **78**: 961-966.

Lucas, J. A., Greer, G., Oudemans, P. V. and Coffey, M. D. 1990. Fungicide sensitivity in somatic hybrids of *Phytophthora capsici* obtained by protoplast fusion. *Physiol. Mol. Plant Pathol.* **36**: 175-187.

Lynch, D. T., Issac, S. and Collin, H. A. 1989. Uptake of protoplasts from the filamentous fungi *Aspergillus nidulans* and *Fusarium oxysporum* into protoplasts from celery (*Apium graveolens* L.). *Protoplasma*

150: 9-18.

Nam, C. G. 1988. Protoplast formation and pathogenicity of regenerated isolates of *Phytophthora capsici* on red pepper. M. S. Thesis, Dongguk University.

Novaes-Ledieu, M. and Garcia-Mendoza, C. 1970. Biochemical studies on walls synthesized by *Candida utilis* protoplasts. *J. Gen. Microbiol.* **61**: 335-342.

Peberdy, J. F. 1979. Fungal protoplasts: Isolation, reveration, and fusion. *Ann. Rev. Microbiol.* **33**: 21-39.

Peberdy, J. F. and Ferenczy, L. 1985. Fungal protoplast. New York, U.S.A.: Marcel Dekker Inc.

Peberdy, J. F. and Hocart, M. J. 1987. Protoplasts as a tool in the genetics of plant pathogenic fungi. pp. 127-141. In: Genetics and Plant Pathogenesis. P. R. Day and G. J. Jellis, Eds. Black Well Scientific Publications, Oxford.

Tanaka, H., Ogasawara, N. and Kamimiya, S. 1981. Protoplasts of *Pyricularia oryzae* P_2: Preparation and regeneration into hyphal form. *Agric. Biol. Chem.* **45**: 1541-1552.

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