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Protoplast Formation and Fusion between Anastomosis Groups of *Rhizoctonia solani*

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Rhizoctonia solani 融合群 間의 原形質體形成 및 融合

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ABSTRACT: The protoplast formation of *Rhizoctonia solani* in the fast growing anastomosis groups (AGs) 1 and 4, the intermediate AG-2 and AG-5, and the slow AG-3 yielded the most, moderate and the least in that order, respectively. Sclerotia formation varied with AGs. A high yield of protoplasts from AGs was obtained with a combined lytic enzyme system containing cellulase "Onozuka" R-10, macerozyme R-10 and β-glucuronidase. When 3g (fresh weight) of 30 hr old mycelia was incubated for 3 hr at 32°C with the enzyme mixture in 0.6 M mannitol, maximum protoplasts were obtained in the five AGs. A protoplast fusion between sclerotia forming AG-1 inactivated with heat and non-forming AG-5 was induced by polyethylene glycol and Ca^{2+}. Seven fusants obtained were based on characteristics of colony and sclerotium formation on culture plates. The fusants were confirmed by isozyme patterns of esterase and killing reaction between AG-1 and a fusant F1501.

KEYWORDS: *Rhizoctonia solani*, Anastomosis groups, Protoplast formation, Protoplast fusion.

Rhizoctonia solani Kühn, teleomorph *Thanatephorus cucumeris* Donk, is a cosmopolitan fungus in soils but its sexual form is rarely observed in the nature. This fungus is a facultative pathogen which grows fast and colonizes aggressively on organic debris. As a destructive plant pathogen with almost unlimited host range, it causes sheath blight of rice, damping-off, root rot, crown and stem rot, and occasionally foliar blight (Adams, 1988; Ogoshi, 1987). There are over 30 plant species reported as the hosts of *R. solani* in Korea (Anonymous, 1986).

The wide host range and various symptoms resulted in difficulty to develop the efficient resistant cultivar against the diseases caused by *R. solani* (Adams, 1988). The species, *R. solani*, was not able to be easily subdivided at the subspecies level because of its variation in pathogenicity, morphology, physiology, ecology, and genetic traits (Parmeter *et al.*, 1970). Recently, the classification of *R. solani* based on hyphal anastomosis between its isolates was widely accepted with the experimental supports showing consistancy among its anastomosis groups (AGs) (Kuninaga *et al.*, 1980; Ogoshi, 1987; Reynolds *et al.*, 1983).

Recently, the fungal protoplast has been focused physiological, biochemical, and genetic studies (Peberdy *et al.*, 1985). Hashiba *et al.* (1982) reported protoplast formation and fusion in anastomosis group 1 (AG-1) of *R. solani*. However, there was no attempts to compare protoplast yields among different AGs and their fusion between AGs made. Protoplast yield might be varied with the AGs under the specific conditions and protoplast fusion between the AGs might be lead to better understanding of taxonomic relations of *R. solani*. Attempts were made to improve protoplast yields among the five AGs and to induce protoplast fusion bet-

ween the AGs, to understand taxonomic relations as well as to apply for control measures of *Rhizoctonia* diseases.

Materials and Methods

Anastomosis groups and culture media: The five anastomosis groups (AGs) of *Rhizoctonia solani* Kühn used in the present work were obtained from the Korean Ginseng and Tobacco Research Institute. Cultures were maintained on potato sucrose agar (PSA; 200g potato, 20g sucrose, 20g agar in the 1000 m*l* water) at the room temperature. In every experiment, mycelial fragments were precultured on PSA for 2 days at 25℃ and marginal culture was used as an inoculum. Growth rate and sclerotia formation of the five AGs were examined on PSA, and modified Czapeck Dox (CD I, CD II) at 20℃ for 5 days. Modified Czapeck Dox agar (CD I) consisted of 5g peptone, 1.5g yeast extract, 15.0g sucrose, 1.0g $NaNO_3$, 1.0g K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.5g KCl, 0.01g $FeCl_3 \cdot 6H_2O$, 0.01g $CuSO_4 \cdot 5H_2O$, 0.01g $ZnSO_4 \cdot 7H_2O$, and 20g agar in 1000 m*l* water. Czapeck Dox agar (CD II) does not contain peptone and non yeast extract.

Protoplast formation: Protoplast formation from the five AGs was based on the modified method of Hashiba *et al.* (1982) and Lee *et al.* (1985). Fresh mycelium cultured statically in 50 m*l* of PS broth at 25℃ were harvested through the filter paper and washed twice with the sterilized water. The mycelium was suspended in the 10 m*l* enzyme solutions at 32℃. Optimum ranges of culture age, fresh weight of mycelium and incubation time in the lytic enzyme solutions were compared in the five AGs. The culture was filtered through a sintered glass filter (pore size 20-30 μm) to remove mycelial fragments, and the filtrate was centrifuged at least twice at the 1000g for 5 min, to remove the enzymes.

The enzyme solutions to release protoplasts contained cellulase "onozuka" R-10 (20 mg/m*l*) from *Trichoderma viride*, macerozyme R-10 (5 mg/m*l*) from *Rhizopus* sp., driselase (10 mg/m*l*) from Basidiomycetes, and β-glucuronidase Type H-2 (0.06 mg/m*l*) from *Helix pomatia*, in 0.6 M mannitol. These enzymes were sterilized by passing them through the membrane filter. The enzymes were tested singly or in combinations.

The pellet obtained consisted of intact protoplasts, broken protoplasts, undigested tissues, and cellular organelles. The pellet was resuspended in the 0.5 m*l* of 0.6 M mannitol. The liquid-liquid two-phases system of 0.6 M sucrose and 0.6 M mannitol used by Hashiba *et al.* (1982) was applied for the separation of intact protoplasts. The protoplast suspension was gently layered onto 1 m*l* of 0.6 M sucrose in Effendorf tube, and centrifuged at 200g for 5 min at the room temperature. The intact protoplasts at the interphase removed with micropipette were resuspended and centrifuged at 1000g for 5 min.

Mannitol, sucrose, potassium chloride, and magnesium sulfate were studied to choose a proper osmotic stabilizer. The concentration of each stabilizer was adjusted to 0.6 M with the distilled water (pH 7.0).

Protoplast fusion: Sclerotium formation in the AG-1 and no sclerotium in the AG-5 were used as a marker to select the protoplast fusants after the process of protoplast fusion. To determine the initial inactivation temperature, the AG-1 protoplasts made were heat-treated in a water bath at 55℃ for 0, 5, 10, 15, 20 min respectively, flooded on the solid regeneration media, and then incubated at 25℃ for 5 days. The temperature showing no visible colony was considered to be the thermal inactivation point of protoplasts.

Protoplasts between the AG-1 and the AG-5 were fused by modifying the method of Hashiba *et al.* (1984). Protoplasts obtained from the heat inactivated AG-1 and AG-5 to be fused were mixed and centrifuged at the 3500g for 5 min. The pelleted protoplasts were resuspended in the 0.4 m*l* of a solution containing 40% polyethylene glycol (PEG) and $CaCl_2 \cdot H_2O$ in 10 mM tris-HCl buffer and adjusted to pH 7.0 with KOH. After incubation for 20 min, the suspension was centrifuged at 3500g for 5 min. Protoplasts from fusion mixtures were suitably diluted and plated on PSA supplemented with the 0.6 M mannitol solutions.

Isozymes of esterase: Samples containing proteins of *R. solani* were prepared from PSB-grown cultures. Mycelia were harvested on filter paper after 7 days incubation at 25℃. The homogenates were centrifuged at 15000 rpm and supernatant was uaed for electrophoresis of esterase.

Electrophoresis of the proteins in the cell free extracts of mycelium was performed with the 10% gel system consisting of 5% methylene-bis acrylamide cross-linking agent in 95% acrylamide monomer in 0.1 M tris-HCl buffer at pH 7.5 (Matsuyama *et al.*, 1978). N,N,N′,N′-tetramethylene-diamine (0.15%, v/v) was used as a gelling primer and 0.1% (w/v) ammonium persulfate as a gelling accelerator. The prepared 250 μg protein samples were applied in each well with micropipette. Electrophoresis was performed at 4℃ for 5 hr. Voltage was adjusted to 200 V and starting current was between 20 and 30 mA, decreasing to 10 mA at the end. After electrophoresis, gels were stained for esterase. Acidity of the gel was adjusted with 0.1 M tris-HCl buffer (pH 7.2) before esterase staining, and the gel was transferred to staining solution containing 60 mg α-naphthylacetate and 70 mg Fast Blue RR salt in 120 m*l* 0.1 M tris-HCl buffer. Destaining was performed electrophoretically in 2% acetic acid at room temperature for 120 min.

Table 1. Growth rates of five anastomosis groups of R. solani on different media incubated for 3 days at 25℃.

Anastomosis Group	Colony diameter (mm)/Day[a]		
	PSA[b]	CD I[c]	CD II[d]
AG-1	30	25	23
2	23	21	20
3	11	10	09
4	30	29	27
5	21	22	17

[a] Based on an average of 3 replications with 5 petri dishes after 3 days, incubation.
[b] Potato sucrose agar.
[c] Modified Czapeck Dox medium I containing 15.0g sucrose, 1.0g sodium nitrate, 1.0g dipotassium phosphate, 0.5g magnesium sulfate, 0.5g potssium chloride, 0.01g ferric chloride, 0.01g cupric sulfate, and 20g agar per 100 m*l*.
[d] Modified Czapeck Dox medium II without both peptone and yeast extract in CD I.

Result

Cultural characteristics of anastomosis groups (AGs): My celial growth and sclerotium formation of each AG were compared on potato sucrose agar (PSA) and modified Czapeck Dox agar (CD I, CD II) at 25℃ for 72 hr (Table 1). AG-1 and AG-4 grew fast and those covered the plates of 90 mm in their colony diameter within 72 hr on PSA. AG-2 and AG-5 the intermediate and AG-3 the slowest reached to 34 mm in diameter on PSA. In general, PSA was the best and followed by modified Czapeck Dox agar I and II regardless of AGs tested.

Sclerotia were formed in AG-1 and AG-2 on the solid media tested, while no sclerotia were found in AG-3, AG-4, and AG-5. Sclerotia of AG-1 were grain form and those of AG-2 were sand form on the circle line of the plates. Sclerotia were found 3 and 4 days after incubation of AG-1 and AG-2, respectively, on each medium. The morphology of sclerotia in AG-2 varied with each medium tested.

Factors affecting protoplast formation

Lytic enzyme: As a single enzyme β-glucuronidase was the most effective for protoplast production from AG-4 (1.2×10^7/g), and the next was cellulase "onozuka" R-10 (1.0×10^4/g) (Table 2). No protoplast was observed with macerozyme R-10

Table 2. Protoplast yields from R. solani AG-4 in different enzyme combinations in 0.6 M mannitol, when incubated at 32℃ for 3 hr

Enzyme combination	Protoplast yield[a]
Cellulase "onozuka" R-10, A	1.0×10^4
β-glucuronidase, B	1.2×10^7
Macerozyme R-10, C	0
Driselase, D	0
A+B	6.5×10^7
A+B+C	8.4×10^7
A+B+C+D	8.5×10^7

[a] Counted with hemocytometer from 1 g or fresh culture

Table 3. Effect of osmotic stabilizers on protoplast yield[a] and regeneration of protoplasts in Rhizoctonia solani AG-4

Osmotic stabilizer[b]	Protoplasts released[c] /g dry mycelium	Regeneration frequency(%)[d]
Mannitol	8.0×10^7	25.0
Potassium chloride	8.7×10^7	8.5
Magnesium sulfate	1.0×10^6	22.5
Sucrose	5.0×10^5	19.5

[a] Prepared after treatment at 32℃ for 3 hr in enzyme combination containing cellulase "onozuka" R-10, β-glucuronidase, and macerozyme. [b] The concentration was 0.6 M in 10 ml of each osmotic stabilizer.
[c] Counted with hemocytometer.
[d] (Number of visible colonies on regeneration medium/number of protoplasts flooded)×100.

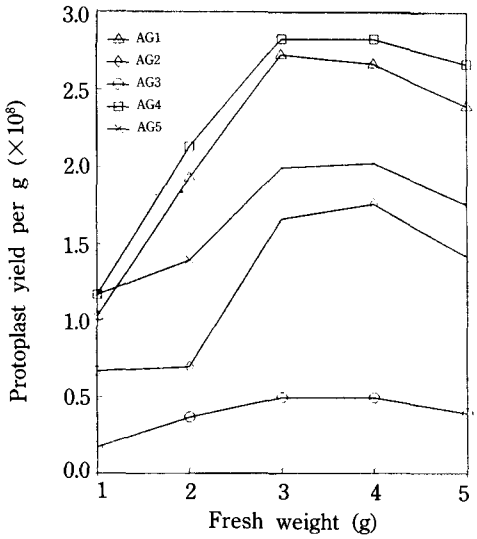

Fig. 1. Effect of fresh mycelial weight on protoplast yields on different amount of mycelium when five *Rhizoctonia solani* AGs mycelia were incubated for 3 hr at 32℃ with enzyme mixture in 0.6 M mannitol.

or driselase. Enzyme mixture with β-glucuronidase, cellulase "onozuka" R-10, and macerozyme R-10 were significantly active and yielded 8.5×10^7 protoplasts/g mycelia.

Osmotic stabilizer: Among the candidates of osmotic stabilizers mannitol and potassium chloride were more effective than the others. These produced 8.0×10^7 and 8.7×10^7 protoplasts/g mycelium in 0.6 M, respectively (Table 3). The same concentrations of magnesium sulfate and sucrose also produced 1.0×10^6 and $5.0\times/10^5$ protoplasts/g mycelium, respectively.

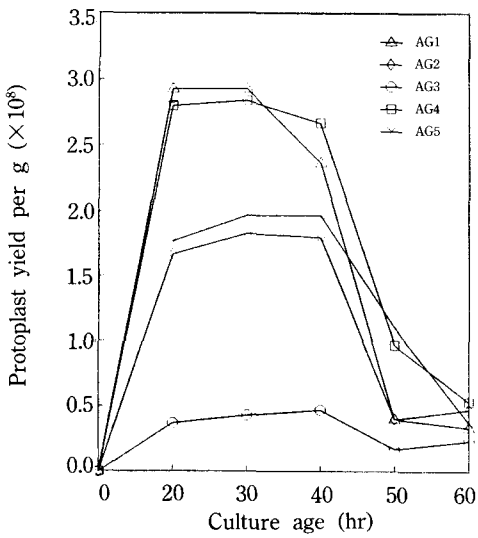

Fig. 2. Effect of mycelial age on protoplast yields when 3 g of five *Rhizoctonia* AGs mycelia were incubated for 3 hr at 32℃ with the enzyme mixture in 0.6 M mannitol.

Amuont of fresh mycelium: Protoplast yields varied with amount of fresh mycelium of the five AGs when mycelium was reacted with the lytic enzymes. All the AGs resulted in the most protoplasts from 3.0-4.0g of mycelium. When the weight was above 4.0g or below 3.0g, the yields were decreased (Fig. 1).

Culture age: Mycelia from cultures of different ages were tested for protoplast production. The age of mycelium greatly affected the yield of protoplasts. Yields were greatly reduced when the mycelium had been grown for more than 50 hr (Fig. 2). Although protoplast yields were different from the five AGs, the optimum range of mycelial age was 30-40 hr.

Time of enzyme treatment: The maximum release of protoplast in the five AGs was obtained after incubation for 2.5-3.5 hr with enzyme mixtures. Prolonged treatment beyond 3.5 hr did not increase the yield of protoplasts (Fig. 3).

Prptoplast regeneration: Mannitol, magnesium

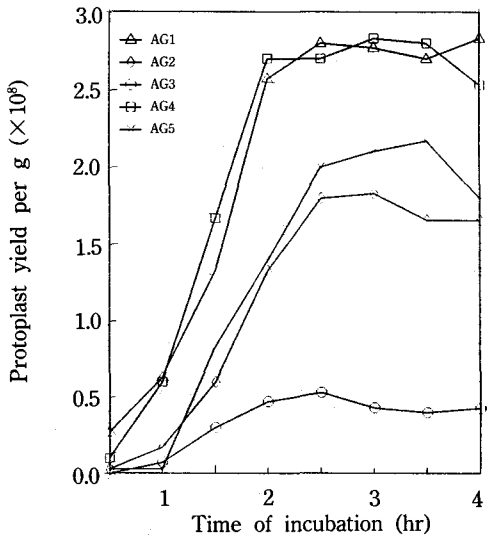

Fig. 3. Time course of the release of protoplasts from mycelia of the five AGs of *Rhizoctonia solani* when 3g of five AGs mycelia were incubated for 3 hr at 32℃ with the enzyme mixture in 0.6 M mannitol.

Table 4. Effect of heat treatment[a] on protoplast regeneration[b] of Rhizoctonia solani

Period of heat treatment(min)	Colony number[c] regeneration per plate
0	200
5	45.3
10	12.5
15	0
20	0

[a] Heat treated with 1 ml protoplast suspension in a water bath by gentle hand-shaking.
[b] Regeneration on PSA osmotically stabilized with 0.6 M mannitol.
[c] Counted after 5 days at 25℃.

sulfate, and sucrose in 0.6 M were proved to be effective osmotic stabilizers for regeneration and regeneration percentages were 25.0, 22.5, 19.8%, respectively (Table 3). However, potassium chloride which was effective for the protoplast production was the least for regeneratoin.

Protoplast fusion: Sclerotia formation in AG-1 and no sclerotia in AG-5 on all the media was used as a selection marker of protoplast fusants. The AG-1 was inactivated by heat treatment in a water bath to be used for protoplast fusion.

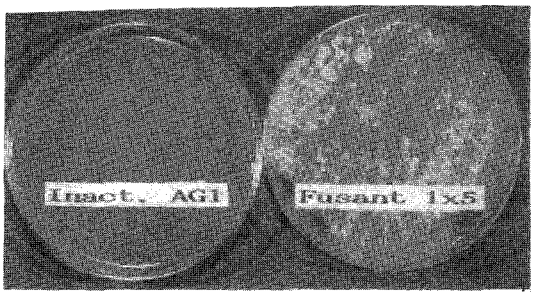

Fig. 4. Protoplast fusants between heat inactivated AG-1 and viable AG-5 of *Rhizoctonia solani* on potato sucrose agar for 10 days at 25C.

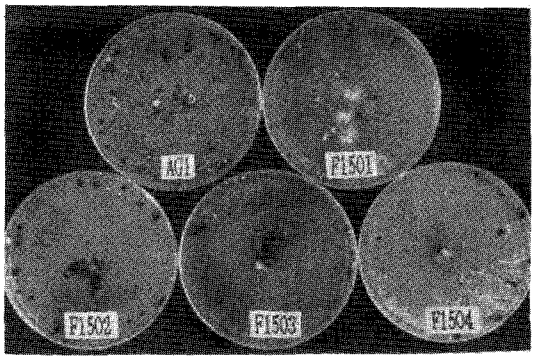

Fig. 5. Characteristics and sclerotium morphology of protoplast fusants between AG-5 and heat inactivated AG-1 of *Rhizoctonia solani* grown on potato sucrose agar at 25C.

When protoplast suspension was treated at 55℃ for 15 min, no colony appeared on the regeneration media. This was considered thermal inactivation point for protoplasts (Table 4).

Protoplasts of sclerotia forming AG-1 and not forming AG-5 were fused with PEG and Ca^{2+} treatment . Between heat inactivated AG-1 and viable AG-5 formed sclerotia on the fusion plate (Fig. 4). Seven fusants obtained were different from their parents in their colony and sclerotium morphology (Fig. 5). Killing reaction was observed between AG-1 and F1501 fusant between AG-5 and heat inactivated AG-1 of *Rhizoctonia solani.*

Isozyme patterns of esterase: Zymogram patterns of esterase in parental AGs and their fusants were different. In the fusant F1501, a new band which could not be overserved in the parental AGs were found and the amounts of proteins varied with AGs and the fusant (Fig. 7).

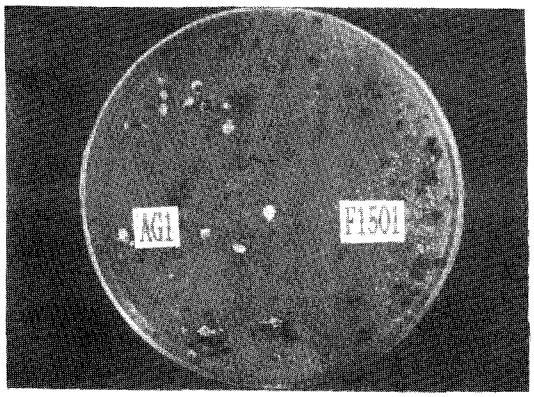

Fig. 6. Killing reaction between the AG-1 and the fusant F1501 from AG-5 and heat inactivated AG-1 of *Rhizoctonia solani*.

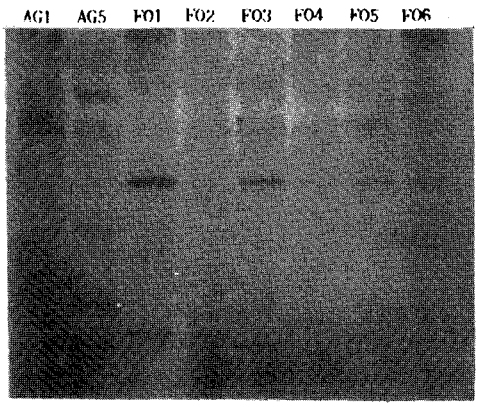

Fig. 7. Isozyme patterns of esterase from protoplast fusants and their parental AG-1 and AG-5 of *Rhizoctonia solani*.

Discussion

The five anastomosis groups (AGs) of *Rhizoctonia solani* were characterized as fast, intermediate and slow growing on various culture media tested (Table 1). The fast growing AG-4 and AG-1 produced the most protoplasts when proper amount of mycelium, culture age and incubation time with the lytic enzymes throughout the experiment and those were followed by moderate in intermediate AG-5 and AG-2 and the least in slow growing AG-3. Although there are many factors influencing the fungal protoplast formation (Davis, 1985), growth rate of *R. solani* might be one of the most useful criteria to get a high yield of protoplasts.

The most effective single enzyme for protoplast production from *R. solani* was β-glucuronidase. However, enzyme combinations resulted in more protoplasts than did with single enzyme. The effect of the combination of these enzyme mixture in enhancing protoplast formation was presumed to result from synergistic action of the major components.

In many cases of protoplast fusion, chemical resistant mutants and auxotrophic or other defective mutants are used as fusion partner (Bradshaw *et al.*, 1983; Ha *et al.*, 1991; Hashiba *et al.*, 1984; Toyama *et al.*, 1984). But obtaining stable marker in sufficient quantity is rather laborous and time consuming. An approach that overcomes this problem was to inactivate the prototrophic partner, sclerotia forming AG-1, and it could be tested with non-forming AG-5 for complementation capabilities as Fodor *et al.* (1978) and Choi *et al.* (1988). This method would be very effective to obtain intra- or interspecific hybrids of the fungi through protoplast fusion when few markers or few marked strains are availible.

Sclerotia were formed on the regeneration medium after fusion process between protoplasts of AG-5 and the inactivated AG-1. The morphology of seven fusants was different from that of parents when they were cultured on PSA again. Isozyme patterns of esterase were also different from each parentral AG and their fusants. Furthermore killing reaction was observed between AG-1 and the fusant F1501. It means that the F1501 involves characteristics of AG-1 in anastomosis however, the fusant F1501 differed from the parent AG-1. Killing reaction is one of the reactions that the same AG may show. This reaction is assumed to be resulted from cytoplasmic incompatibility between isolates within an AG (Adams, 1988).

We have improved a practical high yielding method of protoplasts from five AGs of *R. solani* with a combined lytic enzymes varying with incubation time, amount of mycelium, mycelial age, and proper stabilizers. The fusants were obtained readily from AGs between heat inactivated and viable ones by the presence of sclerotia as a genetic ma-

rker. Fusion between one of each AG indicates an incompatibility in AGs and that a little taxonomic relationship exists between AGs. However, further studies are required to add fusion markers such as complementary auxotrophs and chemical resistant AGs with many isolates.

摘 要

*Rhizoctonia solani*의 균사융합군(AG)중에서 생장속도가 빠른 AG-1과 AG-4는 가장 많은 원형질체를 형성하였으며 속도가 느린 AG-3은 저조하였고 중간인 AG-2와 AG-5는 또한 중간이었다. 균핵의 형성은 AG에 따라 차이가 있었다. Cellulase "onozuka" R-10, macerozyme R-10, β-glucuronidase의 효소 복합액에서 가장 많은 원형질체를 형성하였다. 5개 융합군은 30시간 배양한 3g의 균사를 0.6 M mannitol이 첨가된 효소 복합액에 32℃에서 3-4시간 처리하면 가장 많은 원형질체를 형성하였다. 균핵을 형성하는 AG-1을 열처리로 불활성화시킨 후 균핵을 형성하지 않는 AG-5의 원형질체를 PEG와 Ca^{2+}로 융합시켰다. 재생배지상에서의 균총의 특징과 균핵의 형성에 의해 7개의 원형질 융합체를 선발하고 esterase 동위효소의 zymogram 및 AG-1과 융합체인 F1501 사이에서 일어나는 killing reaction으로 확인하였다.

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