Intraspecific Protoplast Fusion Between Auxotrophic Mutants of *Rhizoctonia solani*

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The authors wish to sincerely thank T. Ishikawa, University of Tokyo, for helpful suggestions and encouragement; H. Yaegashi, of the National Institute of Agricultural Sciences, for kindly offering help; and R. C. Staples, Boyce Thompson Institute, Ithaca, NY, for critically reading the manuscript.

Accepted for publication 23 September 1983.

ABSTRACT

Hashiba, T., and Yamada, M. 1984. Intraspecific protoplast fusion between auxotrophic mutants of *Rhizoctonia solani*. Phytopathology 74:398-401.

Auxotrophic mutants of *Rhizoctonia solani* isolate 131, from anastomosis group 1, were isolated by treatment of protoplasts with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Low-speed centrifugation of protoplast suspensions permitted separation of uninucleate and enucleate protoplasts from predominantly multinucleate protoplasts. Intraspecific protoplast fusion between auxotrophic mutants obtained from the uninucleate/enucleate fraction was induced by polyethylene glycol and Ca^{2+}. The fused cells grew stably on minimal medium and produced sclerotia. Thus, the proposed method is expected to be useful for the isolation of auxotrophic mutants in multinucleate filamentous fungi which produce no spores on artificial media.

The morphological and biochemical characteristics associated with differentiation of sclerotia of the most commonly encountered fungal pathogen in rice plants, *Rhizoctonia solani* Kühn, have been studied extensively (9,11–13), but few studies on genetic manipulation of the pathogen have been reported.

There are many reports concerning intra- and interspecific protoplast fusion between mutants of filamentous fungi including *Aspergillus* (6,7,16) and *Penicillium* (2,3). Several procedures for producing fungal mutants are known, but the production of auxotrophic mutants from *R. solani* anastomosis group 1 (AG-1) (17,18) is difficult because cultures of *R. solani* often fail to sporulate on artificial media and because vegetative cells are multinucleate (8,19).

Recently, we developed a method to isolate and culture protoplasts from *R. solani* (10,14). The present study reports the isolation of auxotrophic mutants of *R. solani* and intraspecific protoplast fusion between those mutants.

MATERIALS AND METHODS

Organisms. *Rhizoctonia solani* Kühn isolate 131, belonging to AG-1, was obtained from an infected rice plant, as described previously (9,12). Cultures were maintained on potato-sucrose agar (PSA).

Protoplast formation. Protoplasts were prepared according to the method of Hashiba and Yamada (14). One gram (fresh weight) of the mycelium grown for 15 hr in 20 ml of potato-sucrose liquid medium containing polypeptone was harvested on a 150-μm steel sieve, washed with distilled water, and suspended in 10 ml of the enzyme mixture containing cellulase "Onozuka" RS (Yakult Biochemical Co., Ltd., Nishinomiya, Japan), 20 mg/ml; Macerozyme R-10 (Yakult Biochemical Co., Ltd.), 5 mg/ml; and β-glucuronidase 0.06 ml/ml (14) in a 100-ml Erlenmeyer flask. The flask was placed on a reciprocal shaker at 75 strokes (4 cm) per min at 34 C for 3 hr. The culture was filtered through a 150-μm steel sieve to remove mycelial fragments, and the filtrate was centrifuged in 0.6 M mannitol at least twice at 700 *g* for 5 min to remove the enzymes. Intact protoplasts were further separated from mycelial fragments and cell debris by an aqueous two-phase system which consisted of 0.6 M sucrose–0.6 M mannitol. Two milliliters of the protoplast suspension were layered onto 4 ml of 0.6 M sucrose, 1 mM $CaCl_2$, 5 mM HEPES-KOH (pH 7.0) and centrifuged in a swinging-bucket rotor at 200 *g* for 5 min at room temperature. The intact protoplasts were located at the interphase of the two-phase system.

Media. To isolate mutants, YS agar medium containing 10 g of yeast extract, 30 g sucrose, 370 mg $MgSO_4 \cdot 7H_2O$, 170 mg KH_2PO_4, 1,900 mg KNO_3, 8.5 mg $ZnSO_4 \cdot 7H_2O$, 27.5 mg $FeSO_4 \cdot 7H_2O$, and 20 g of agar per liter, pH 6.5, was used as the complete medium (CM), and CM without yeast extract was used as the minimal medium (MM). Twenty milliliters of agar medium was used per 9-cm-diameter petri dish.

Mutagenesis and isolation of auxotrophic mutants from protoplasts. Protoplasts of parental strains (5×10^6 protoplasts per milliliter) in 0.05 M phosphate buffer (pH 7.2) supplemented with 0.6 M mannitol were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) for 45–60 min at 25 C to give a 3–20% survival rate. The concentration of NTG used was 20 μg/ml. The mutagenized protoplasts were centrifuged and the pellet washed with mannitol-buffer solution before being suspended in fresh mannitol-buffer. The protoplasts were plated on CM supplemented with 0.6 M mannitol. Colonies that appeared after incubation for 5 days at 28 C were transferred to fresh CM plates, and the resulting colonies were checked for growth on MM. Isolates showing growth on CM but not on MM were scored as auxotrophs. Specific nutritional requirements of auxotrophs were determined by the methods of Holling (15).

Protoplast fusion. Protoplasts were fused by using modifications of the methods of Anné and Peberdy (3), and Ferenczy et al (5). After being washed in 0.6 M mannitol, protoplasts obtained from the two different auxotrophic mutants to be fused were mixed (2×10^6 protoplasts per milliliter of each auxotroph) and centrifuged (3,500 *g*, 5 min). The pelleted protoplasts were resuspended in 0.4 ml of a solution containing various concentrations of polyethylene glycol (PEG) and $CaCl_2 \cdot H_2O$ in 10 mM tris-HCl buffer and adjusted to pH 7.5 with 0.01 M NaOH. After incubation for 15 min, the suspension was centrifuged for 5 min at 3,500 *g*. Protoplasts from fusion mixtures were plated at suitable dilutions on MM and CM supplemented with 0.6 M mannitol. Colonies that appeared after 1 wk of incubation at 28 C were counted. The fusion frequency was calculated from the ratio of colonies growing on MM to colonies growing on CM.

Nuclear staining. Protoplasts were dropped on glass microslides and allowed to dry quickly on a flame. The specimen was fixed in ethanol/acetic acid (3:1, v/v) for 10 min, dehydrated through an ethanol series, and stained with HCl-Giemsa by using a modified method of Colotelo and Grinchenko (4).

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Replication. The data represented in all tables and figures are the average of three experiments, each replicated twice.

RESULTS

Number of nuclei per cell. Hyphal cells of *Rhizoctonia solani* isolate 131 grown on PSA contained an average of 11 nuclei (Fig. 1). When protoplasts were obtained from *R. solani* and centrifuged at 700 *g* for 5 min as described by Hashiba and Yamada (14), protoplasts in the pellet contained less than eight nuclei per cell. Nearly 20% of these protoplasts contained a single nucleus per cell, but other protoplasts contained two or more nuclei per cell (Fig. 1). All protoplasts in the supernatant fraction contained less than four nuclei per cell. More than 60% of these protoplasts were without a nucleus, and 26% of them contained a single nucleus per cell (Fig. 1). The results indicated that most protoplasts in the supernatant fraction were either mononucleate or enucleate. Therefore, protoplasts in the supernatant fraction subsequently were exposed to mutagens and tested for the presence of auxotrophic mutants.

Isolation of auxotrophic mutants. Protoplasts were exposed in buffer to 20 μg of NTG/ml for 60 min, and plated on CM supplemented with 0.6 M mannitol. Survivors were isolated on fresh CM and tested for growth on MM. In tests of 2,351 isolates, two auxotrophic mutants were found, one requiring methionine (met^-) and the other requiring arginine (arg^-) (Fig. 2). The reversion frequency of these mutants was about 10^{-3}.

Conditions for protoplast fusion. Protoplasts were prepared from the met^- and arg^- mutants. The mixture of washed protoplasts of both mutants was centrifuged and suspended in solution containing PEG 4,000 at various concentrations with 10 mM $CaCl_2$. After incubation for 15 min at room temperature, the mixture of protoplasts was plated directly on a selective regeneration medium lacking growth factors (methionine and arginine) required by each parental auxotrophic strain. The frequency of prototrophic colonies was calculated from the ratio of the number of colonies on the MM to that on the CM.

The effect of PEG concentration on the frequency of prototrophic colonies is shown in Fig. 3. High frequencies of prototrophic colonies in these experiments were obtained with 40–60% PEG. Incubation of protoplasts with PEG at concentrations <20% caused protoplast lysis.

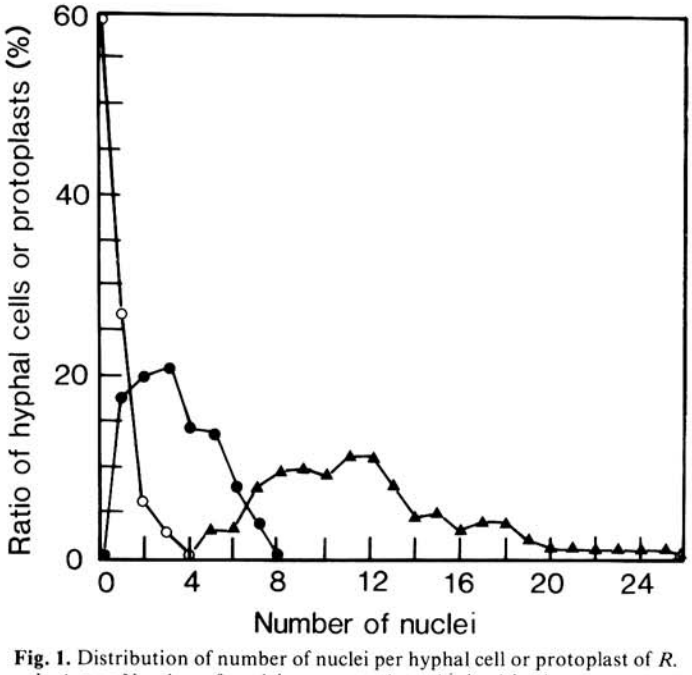

Fig. 1. Distribution of number of nuclei per hyphal cell or protoplast of *R. solani*. o = Number of nuclei per protoplast obtained in the supernatant fraction prepared after centrifugation at 700 *g* for 5 min, ● = number of nuclei per protoplast obtained in the pellet, ▲ = number of nuclei per hyphal cell.

Mixtures of the protoplasts of the mutants were treated with 40% (w/v) solutions of PEGs having molecular weights of 1,540, 4,000, or 6,000 and with $CaCl_2$ concentration at 10 mM. Similar recoveries of prototrophic colonies were obtained across the wide range of PEG tested (1,540 to 6,000) (Table 1). Therefore, use of the intermediate PEG 4,000 is a justified choice.

Table 2 reveals the effect of concentrations of $CaCl_2$ on the frequency of prototrophic colonies from fusion of protoplasts of

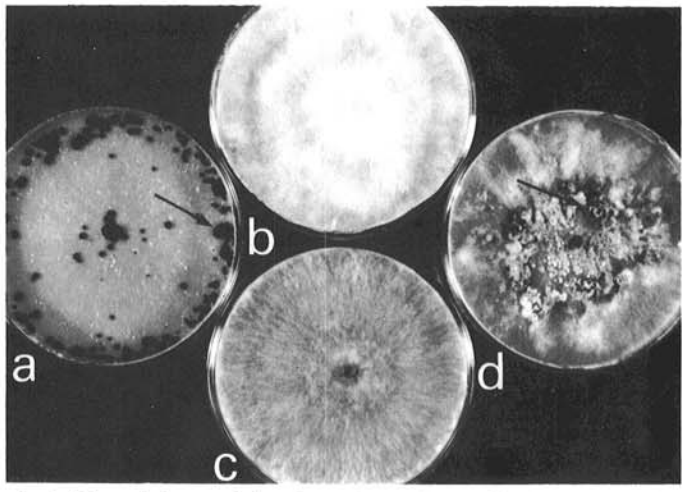

Fig. 2. Cultural characteristics of *R. solani*. **a,** Parent field isolate No. 131 grown on minimal medium (MM). **b,** arg^- mutant grown on minimal medium (MM) supplemented with 20 μg arginine per ml. **c,** met^- a mutant grown on MM supplemented with 20 μg methionine per milliliter. Both of the mutants were normally unable to form sclerotia. **d,** Prototrophic colonies resulting from intraspecific protoplast fusion grew on MM. Arrows indicate sclerotia.

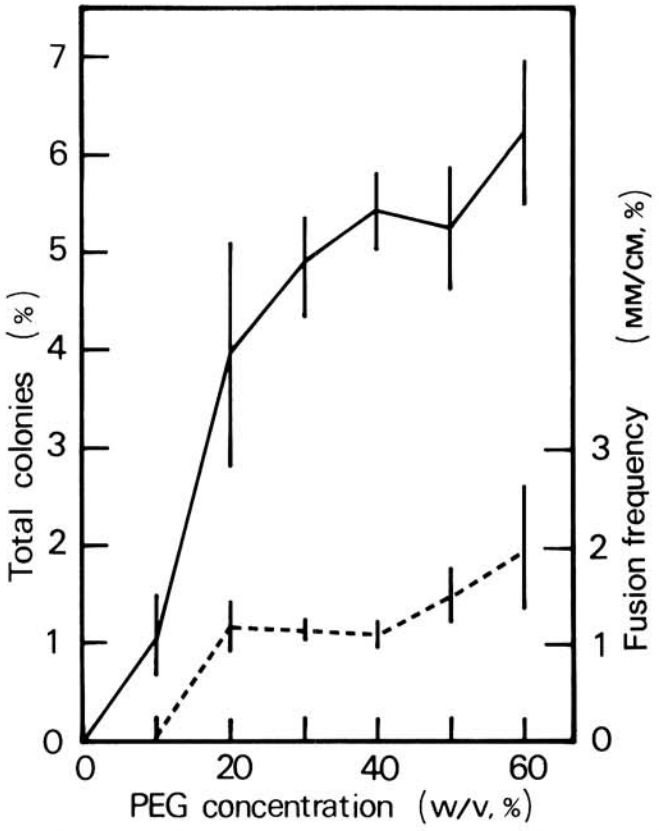

Fig. 3. Effect of PEG 4,000 concentration on frequency of formation of prototrophs. —— = The number of colonies growing on complete medium (CM) divided by the number of protoplasts plated × 100, ---- = the ratio of the number of colonies growing on minimal medium (MM) and CM.

auxotrophic strains. The concentration of PEG 4,000 was kept at 40% (w/v). The number of colonies regenerated on CM increased with increasing $CaCl_2$ concentration. Under the present conditions, intraspecific protoplast fusion between auxotrophic strains gave prototrophic colonies with frequencies of 1–2%. The average frequency of prototrophic colonies against the initial number of protoplast pairs was 2×10^{-3} (Table 2). No colonies appeared from protoplasts that were not fused on MM.

Characteristics of fused protoplasts. Prototrophic colonies were formed on MM following incubation of the fused protoplasts for 5 days. One hundred prototrophic colonies that appeared on the MM were transferred to fresh MM and their properties were examined. As illustrated in Fig. 2, prototrophic colonies differed morphologically from the colonies of the auxotrophic mutant strains and those of the parent field isolate. The parent field isolate produced sclerotia, but auxotrophic mutants produced no sclerotia at all. The colonies regenerated from the fused protoplasts, however, produced sclerotia. Most of them segregated sectors of parental auxotrophs. After the first recultivation of cells originating from fused protoplasts on MM, stable prototrophic progenies were obtained. Intact protoplasts were separated from mycelial fragments of stable prototrophic progenies. Then the fraction with uninucleate and enucleate protoplasts was plated on CM supplemented with 0.6 M mannitol. Colonies were transferred to fresh CM plates, and the resulting colonies were checked for growth on MM. In tests of 73 isolates, all of the isolates grew on MM. The results indicated that the stable isolates that originated from fused protoplasts were prototrophic.

DISCUSSION

Protoplasts have been produced from cells of a large number of fungal species by means of snail digestive juice or enzymes of microorganisms. We had developed methods for protoplast isolation and culture from hyphal cells of *R. solani* (10,13,14). However, several difficulties were encountered in the attempt to isolate auxotrophic mutants of *R. solani*. For example, various isolates of *R. solani* either produced no spores on artificial media or vegetative cells were multinucleate. The present study developed an effective method for obtaining uninucleate protoplasts from multinucleate filamentous cells of *R. solani*.

A schedule for mutagenesis similar to that devised in *Escherichia coli* by Adelberg et al (1) has been followed successfully by many people with a variety of microbes. Colonies originating from regenerated protoplasts that had not been treated with NTG included many morphologically different types. To determine whether the met^- and arg^- mutants could have been "natural" mutants among nuclei in isolate 131, we had previously investigated the frequency of met^- and arg^- mutants in natural populations. In tests of 1,403 isolates, the met^- and arg^- mutants were not found. The results described here indicate that the NTG treatment of protoplasts was effective for the isolation of auxotrophic mutants of *R. solani*. Thus, the proposed method is expected to be useful for the isolation of auxotrophic mutants in multinucleate filamentous fungi which produce no spores on artificial media.

Protoplast fusion has usually been assessed by the detection of genetic recombinants for chromosomal markers. The application of protoplast fusion to isolates of *R. solani* AG-1 for which a sexual cell cycle is not yet known could be promising for genetic studies. Using the auxotrophic mutants described above, we were able to obtain fused cells of *R. solani*. The important factors affecting production of prototrophic colonies appear to be concentrations of PEG and $CaCl_2$ in the fusion mixtures. Below 30% PEG, the frequency of prototrophic colony production fell drastically, while high concentrations of PEG were more effective for the production of prototrophic colonies. Addition of PEG of high molecular weight and high concentrations of $CaCl_2$ favored the production of fused protoplasts. Anné and Peberdy (3) and Ferenzy et al (5) carried out extensive studies of the conditions that influence the efficiency of PEG. They reported that PEG of 4,000 or 6,000 MW were equally effective and that 30% solutions were optimal. The improved survival of *R. solani* at PEG levels higher than 30% may reflect a marked sensitivity of protoplasts of *R. solani* to osmotic damage.

The protoplast mutation and fusion techniques developed in *R. solani* may be broadly applicable for genetic analysis and manipulation of other multinucleate, filamentous, nonspore-producing fungi.

TABLE 1. Effect of molecular weight of PEG on formation of prototrophic colonies

PEG molecular weight[a]	Colony formation per 10^4 protoplasts		Fusion frequency[b] (%)
	CM	MM	
1,540	305	3.7	1.21
4,000	400	4.5	1.13
6,000	472	6.3	1.34

[a] A 40% (w/v) solution of PEG.
[b] Ratio of number of colonies growing on minimal medium (MM) and complete medium (CM).

TABLE 2. Effect of $CaCl_2$ concentration in a solution of PEG 4,000 on the formation of prototrophic colonies by *R. solani*

$CaCl_2$ concentration[a] (mM)	Colony formation per 10^4 protoplasts		Fusion frequency[b] (%)
	CM	MM	
10	366	3.2	0.87
50	1,100	15.9	1.45
100	1,687	23.8	1.41

[a] The PEG 4,000 concentration was kept at 40% (w/v).
[b] Ratio of number of colonies growing on minimal medium (MM) and complete medium (CM).

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