Serological Relationships Among Anastomosis Groups of *Rhizoctonia solani*

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ABSTRACT

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Sixty-four isolates of *Rhizoctonia solani* were compared serologically with the use of antisera developed against one tester strain for each of six anastomosis groups (AG). Gel-diffusion tests showed that all anastomosis groups had several antigens in common; AG-2, AG-3, and AG-5 each had at least one specific antigen. Anastomosis groups 1 and 4 were difficult but possible to separate serologically. The serologic groups corresponded to the anastomosis groups, with the exception of AG-2 type 1 and AG-2 type 2, which were serologically indistinguishable. A rapid, simple method for determining the serologic grouping of field isolates is described. The common antigens were stable when heated 20 min at 121 C.

Additional key words: Ceratobasidium, Thanatephorus cucumeris.

Rhizoctonia solani Kühn (*Thanatephorus cucumeris* (Frank) Donk) is a complex species that has many biotypes differing in pathogenicity, host range, distribution in nature, and appearance in culture. Investigators have attempted (4,5) to assign field isolates to groups according to these characteristics; usually, however, such groups were artificial and contributed little toward understanding the species.

The first natural subdivision of *R. solani* was made in 1936 by Schultz (15), who divided the species into groups according to hyphal anastomosis. His studies were followed by those of Richter and Schneider (13) in 1953, Parmeter et al (11) in 1969, and Ogoshi (7,9,10) in 1972 and 1976. The studies by Schultz (15) and by Richter and Schneider (13) were done before Parmeter and Whitney (12) redefined the species. The fungi placed in group V of Schultz and group E of Richter and Schneider belong in the genus *Ceratobasidium* Rogers.

Parmeter et al (11) recognized four anastomosis groups (AG): AG-1, 2, 3, and 4. Ogoshi (7,9) found these same groups, as well as a new one, AG-5, represented among field isolates from Japan. He pointed out (10) that AG-5 may correspond with group B of Richter and Schneider. Ogoshi (10) subdivided AG-2 into AG-2 type 1 and AG-2 type 2 (AG-6 [7]) according to the infrequency of anastomosis within the group and the cultural characteristics.

The infrequency of hyphal fusion between two isolates often has caused difficulties in tests for anastomosis, especially within AG-2. Field isolates that will not anastomose with any of the established groups also occur (7,9,11). These problems, and the suspicion that isolates that "bridge" the groups eventually might be found, cast some doubt on the utility of anastomosis groups. Nevertheless, the four anastomosis groups, AG-1, 2, 3, and 4, have been discovered independently four times since 1936 by researchers from three continents (7,11,13,15). The groups, in reality, must be biological species.

Biological behavior (ie, anastomosis) has uncovered the natural relationships within *R. solani;* however, we wished to go beyond visual observations to evaluate these relationships. Serologic techniques have distinguished bacterial and fungal subspecies (17), and such techniques might elucidate the similarities and differences among the anastomosis groups on the basis of the chemical homologies of antigenic substances, eg, proteins and glycoproteins. Differences in the antigenic substances among isolates of *R. solani* would suggest genetic distance caused by isolation and drift.

The present study was undertaken to determine if (i) serologic groups correspond to anastomosis groups, (ii) serologic techniques will reveal similarities among isolates within each group, (iii) serologic techniques can be used to assay field isolates easily and quickly for their anastomosis groupings.

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MATERIALS AND METHODS

Isolates. Sixty-four isolates of *R. solani* were collected worldwide. We grouped 25 of these by anastomosis according to the method of Parmeter et al (11); 25 isolates had been grouped previously (11). The standard isolates (tester strains) for testing anastomosis were used to induce antibody formation in rabbits (Table 1): AG-1, isolate 43; AG-2 type 1, isolate 229; AG-2 type 2, isolate 460; AG-3, isolate 141; AG-4, isolate 283; and AG-5, isolate 441. The choice of these isolates was critical to this research. The anastomosis groups AG-1, 2, 3, and 4 are based on these tester strains (11), which have been distributed to many researchers worldwide. Strains of AG-5 and AG-2 type 2 used for immunization were selected from the most vigorous cultures sent to us by A. Ogoshi (National Institute of Agricultural Sciences, Tokyo, Japan). Unfortunately, none of the cultures we received were referred to in his publications.

Antigen preparation. To eliminate any antigenic substances that might be present in natural media, antigens for the immunization of rabbits were prepared from mycelium that had grown after three transfers on a synthetic medium. The synthetic medium (GNA) was composed of D-glucose, 20 g; $NaNO_3$, 20 g; KH_2PO_4, 1.75 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; $CaCl_2 \cdot 2H_2O$, 37 mg; $FeCl_3 \cdot 6H_2O$, 1.0 mg; $ZnSO_4 \cdot 7H_2O$, 0.9 mg; $CuSO_4 \cdot 5H_2O$, 0.8 mg; $Na_2MoO_4 \cdot H_2O$, 0.3 mg; thiamine-HCl, 0.15 mg; agar, 15 g; and distilled water, 1,000 ml. The medium was adjusted to pH 6.5 with 1 N NaOH before autoclaving at 121 C for 20 min. One hundred 1-L Roux bottles containing 60 ml of the synthetic medium without agar (GN) were inoculated with approximately 30 plugs of 1-cm diameter taken from the margin of actively growing cultures of each tester strain. After 3-day incubation at 23–27 C, the mycelium was harvested on cheesecloth and covered with a saline-buffer solution (K_2HPO_4, 0.025 M; KH_2PO_4, 0.025 M; $MgCl_2$, 0.01 M; NaCl, 0.14 M; pH of solution, 7.4). In this instance only, 0.124 N formaldehyde was added to the saline buffer to enhance the antigenicity of the proteins present, and 50 g/L of insoluble polyvinylpolypyrrolidone was added to inactive phenols. The mycelium in the saline-buffer solution was frozen at −20 C, thawed, homogenized in an Omnimixer (Sorvall) for 2 min, and sonicated (Biosonik III, Bronwill Science Co., Rochester, NY 14603) for 10 min at 20 KHz at 5 C; then it was rehomogenized with acid-washed sand for 5 min, refrozen, thawed, and stirred 8 hr at 4 C. The homogenized mycelial suspension was centrifuged at 10,400 *g* for 40 min; the pellet was dis-

carded. The supernatant was placed in a large dialysis tube and covered with hydrophilic polyethylene glycol, 70,000 mol wt, which reduced the volume to approximately 100 ml after 1 wk at 4 C. This concentrate was placed in new tubing and dialyzed against the saline-buffer to remove substances that would interfere with Bradford's (2) method of protein determination. The concentrate was adjusted to 10 mg of protein per milliliter with saline buffer and used as the antigen for immunization.

Each isolate of *R. solani* used in gel-diffusion analysis was grown on GN in three Roux bottles, harvested after 3 days, frozen at −20 C, and crushed in an Aminco-French pressure cell (American Instruments Co., Inc., Silver Spring, MD 20907) at 7.32 kg/mm^2 with an equal volume of saline buffer. The mash was centrifuged and the supernatant was dialyzed against saline buffer and adjusted to a protein concentration of 1.5 mg/ml. This method of antigen preparation was used when small quantities of antigen were needed; the former method was used for bulk preparations.

Immunization methods. Twelve New Zealand White doe rabbits, 5–7 kg, were immunized, two for each tester strain. Two immunization schedules were followed for comparison. In the first schedule, six rabbits, one for each tester strain, were immunized subcutaneously once a week for 4 wk. The first week 2.5 ml of the appropriate antigen preparation blended with 2.5 ml of Freund's complete adjuvant (Difco Bacto H37Ra) was injected. During the next 3 wk, Freund's incomplete adjuvant replaced the complete adjuvant. In the second schedule, the remaining six rabbits were injected each day subcutaneously without adjuvant. The dose of antigen preparation was increased daily by 0.5 ml for 10 days,

TABLE 1. Isolates of *Rhizoctonia solani*: Anastomosis groups (AG), method of antigen preparation, geographic origin, and source or reference

Isolate	AG	Method of antigen preparation	Host	Geographic origin	Source or previous reference
43	1	I[a], F[b], M[c]	*Pinus resinosa* Art.	Quebec, Canada	Parmeter et al (11), R43
65	1	F, M	*Picea glauca* (Moench) Voss.	Quebec, Canada	Parmeter et al (11), C65
189	1	F, M	*Brassica cauliflora* L.	...	ATCC 13248 (11)
199	1	F, M	*Ficus* L.	Louisiana	CMI 61796 (11)
239	1	F, M	*Glycine max* L.	Quebec, Canada	Parmeter et al (11), C239
245	1	F, M	Bean	Costa Rica	Parmeter et al (11), C245
309	1	F, M	*Brassica oleracea* L.	Wisconsin	Parmeter
404	1	F, M	*Oryzae sativa* L.	Louisiana	Bolkan and Butler (1)
465	1	M	*O. sativa*	Japan	Ogoshi, C-54
466	1	F, M	*Raphanus sativus* L.	Japan	Ogoshi, RS-Chi-2
229	2	I, F, M	*R. sativus*	California	Parmeter et al (11), C229
234, 235, 289	2	F, M	...	Australia	Parmeter et al (11), C234, C235, and C289
328	2	M	*Gypsophila* L.	North Carolina	Parmeter et al (11), S284
472 to 474	2	F	*Beta vulgaris* L.	Michigan	Richter and Schneider RS-7421-6, RS-12-10-1, and RS-12-10-2 (13)
455, 457, 461	2-1	F, M	...	Japan	Ogoshi BN-1 (8), PS-2, and BV-42
458	2-1	F, M	*Juncus effusus* L.	Japan	Ogoshi CI-21
454	2-2	F	...	Japan	Ogoshi SA-108-1b
456	2-2	F	*Juncus effusus* L.	Japan	Ogoshi G-6
460	2-2	I, F	*Beta vulgaris* L.	Japan	Ogoshi BV-41
480, 482, 483	2-2	F	Soil	Japan	Ogoshi A-19, H-1, and H-71
481	2-2	F	*Beta vulgaris* L.	Japan	Ogoshi A-83
141	3	I, F, M	*Phaseolus* L.	...	ATCC 14006 (11)
291, 293	3	F, M	*Solanum tuberosum* L.	Ecuador	Parmeter et al (11), C291 and C293
500 to 509	3	F, M	*S. tuberosum*	California	Adams
31	4	F, M	*Beta vulgaris* L.	California	Bolkan and Butler (1)
41	4	M	*Picea glauca* (Moench) Voss.	Canada	Parmeter et al (11), C41
107, 108, 110, 113	4	F, M	*Pinus banksiana* Lamb.	Canada	Bolkan and Butler (1)
112	4	F, M	*Pinus* sp.	Canada	Parmeter
125	4	F, M	*Medicago sativa* L.	Michigan	ATCC 10154 (11)
132	4	M	*Beta vulgaris* L.	Virginia	ATCC 10177 (1)
142	4	F, M	*B. vulgaris*	...	ATCC 14007 (1)
283	4	I, F, M	Conifer	California	Parmeter et al (11), C283
284, 285	4	F, M	*Gossypium hirsutum* L.	California	Parmeter et al (11), C284, C285
286	4	F, M	*G. hirsutum*	Arizona	Parmeter et al (11), C286
313	4	M	*G. hirsutum*	Louisiana	Parmeter
320	4	M	*Pinus palustris* Mill.	North Carolina	Parmeter
436	4	F, M	Bean	California	Butler
440, 462	5	F, M	*Glycine max* L.	Japan	Ogoshi GM-12, Gu-2
441	5	I, F, M	Soil	Japan	Ogoshi Sc-1
463	5	F, M	*Solanum tuberosum* L.	Japan	Ogoshi R-470
464	5	F, M	...	Japan	Ogoshi CC-521-22
477	*Ceratobasidium*	F, M	*Sequoia sempervirens* (D. Don) Endl.	California	Parmeter
478, 479	*Ceratobasidium*	F, M	*Fragaria* L.	California	Adams
495	*Ceratobasidium*	M	*Coleus* Lour.	California	Butler
496	*Ceratobasidium*	M	...	...	Parmeter CH2-26R

[a]I = antigen prepared for immunization.
[b]F = antigen prepared for gel-diffusion analysis with the French press.
[c]M = antigen prepared for field isolate studies with mortar and pestle.

followed by a 4-day interval with no injections, and terminated by a booster of 5 ml. Antisera were collected by cardiac puncture 10 days later.

Serologic techniques. Titers of antisera were determined by double diffusion analysis against the antigens used for immunization, at concentrations of 10 mg protein per milliliter of antigen preparation and 1 mg protein per milliliter of antigen preparation.

Gel-diffusion plates were prepared with 9 ml of 0.9% Ionagar (Colab Laboratories, Inc., Chicago Heights, IL 60411) in the saline buffer with 80% phenol added as a preservative at 0.04 ml/L. Polyethylene glycol (6,000 mol wt) was added at 40 g/L to enhance precipitin band visibility. Soluble antigens and undiluted sera were reacted in a well pattern with six equally spaced outer wells, 14 mm diameter, and one interior well, 17 mm diameter; the center of the interior well was 20 mm from the center of each outer well.

The reactant was added to the wells: 200 μl to each outer well and 300 μl to the central well. Plates were incubated in a moist chamber at 4 C for 7 days. The agar surface then was washed with a 0.25% cadmium acetate solution, to enhance precipitin band visibility, and was incubated 3 days at 4 C.

Sera cross-absorbed with antigens were used to clarify relationships between multiple precipitin band systems. A volume of serum was absorbed with two volumes of antigen, incubated at 37 C for 24 hr, then centrifuged at 1,500 *g* for 30 min. The supernatant was decanted, refrigerated 12 hr, and recentrifuged at 12,000 *g* for 30 min. This supernatant was checked for reaction with the antigen to confirm complete absorption before gel-diffusion analysis.

Rapid assay for grouping field isolates. Serology was evaluated to assess its potential as a rapid method for determining the anastomosis groups of field isolates. Field isolates of *R. solani,* anastomosis groups known and unknown, were grown for three days in 250-ml flasks, each containing 10 ml of GN. Potato-dextrose broth and malt extract broth were equally satisfactory in preliminary tests. The growth media did not alter the precipitin band patterns. The mycelium was harvested on filter paper, placed in 2 ml of saline buffer, and crushed with sand in a mortar and pestle. The crushed mycelial paste of each isolate tested was placed into two outer wells side by side and antiserum was placed in the central well (Fig.1A, B). Three isolates, one a tester strain to provide a control, were tested per gel-diffusion plate. Antiserum of tester strain AG-3 or AG-4 was used in this study because these isolates of *R. solani* were predominant in nearby fields. Thirty-nine isolates of known AG groupings (Table 1) and 10 of unknown groupings were tested by this method. After 48 hr at 25 C the precipitin band patterns were observed.

Antigen composition. To determine if reactive substances other than protein might be present in the antigen preparations, the six antigens used for immunization were autoclaved for 20 min at 121 C before testing. In addition, they were incubated 2 days at 37 C with 10 mg of pronase, grade B (Calbiochem Behring Corp., La Jolla, CA 92037) per milliliter of antigen preparation, then autoclaved to destroy any residual enzyme activity before testing by gel-diffusion analysis.

RESULTS

Immunization methods. The immunization schedule that required daily injections without adjuvant was designed originally for intravenous injections. However, the intravenous injections had to be abandoned and subcutaneous injections substituted after 3 days. The intravenous injections caused gross swelling at the base of the rabbits' ears and occasionally an inflamed swelling of the entire ear; collapse of the marginal ear vein followed, often with necrosis.

Titers. After the initial immunization schedule, antisera titers tested by gel-diffusion ranged from 1/256 to 1/512. The injection schedules did not appear to give antisera that differed in their final titer. After two booster injections, titers approximately doubled. The two antisera for each tester strain were pooled and assayed against antigen preparations of 1 mg of protein per milliliter. These titers averaged 1/64. All antisera were adjusted to this titer for qualitative studies of the relationships among the anastomosis groups.

Common antigens. The antiserum from each of the six tester isolates was tested initially by gel-diffusion against each of the six antigen preparations used for immunization. The antisera were found to give stable precipitin band patterns in seven separate tests. The antisera were then tested in two separate tests against the French press antigen preparation from each of the 64 isolates. All isolates of *R. solani*, including isolates that could not be placed in any anastomosis group, appeared to contain four common antigens, represented by bands 1, 2, 3, and 4 (Fig. 2A), except AG-4, which did not show band 4 (Fig. 3E) and thus had only three common antigens. *Ceratobasidium* spp. shared bands 2, 3, and 4 with *R. solani* (Fig . 2B). Precipitin band system 1 was visually faint in the case of *R. solani*, so the absence of this antigen may be a quantitative artifact.

Distinguishing the anastomosis groups. Anastomosis groups 1, 3, 4, and 5 were differentiated from one another by several unique precipitin bands. Group 2, types 1 and 2, were interpreted to be identical since all antigen preparations of 2-1 and 2-2 isolates gave identical band patterns when compared with all six sera (Fig. 3B, C). However, no bands 5 and 6 were present between the serum of 2-1 and antigens of 2-1 or 2-2 like those present for the serum of 2-2 (Fig. 3B, C). We do not know the reasons for this variable response.

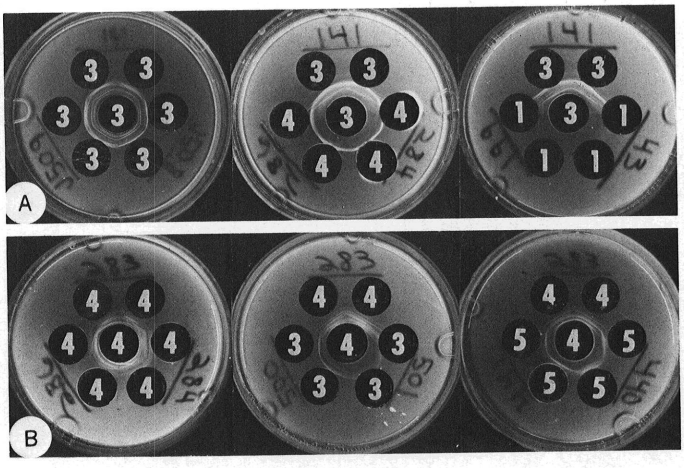

Fig. 1. Representative gel-diffusion tests differentiating field isolates of *Rhizoctonia solani* by their anastomosis group. The central well contains antiserum. Outer wells contain three isolates of *R. solani*, one isolate per two adjacent wells. Numbers in the wells correspond to anastomosis groups. Numbers outside the wells correspond to isolates listed in Table 1. **A)** Central well contains serum of tester strain AG-3; AG-3 isolates are differentiated by multiple precipitin bands. **B)** Central well contains serum of tester strain AG-4; AG-4 isolates are differentiated by multiple precipitin bands.

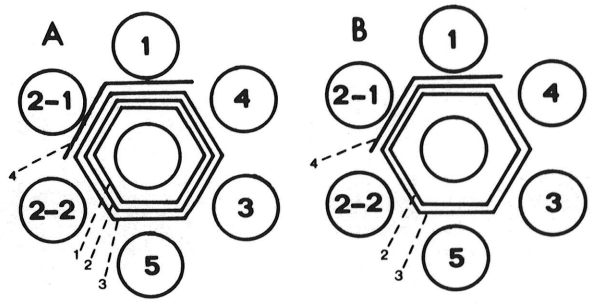

Fig. 2. Diagrammatic representation of gel-diffusion plates showing common precipitin band systems for isolates of *Rhizoctonia solani* and *Ceratobasidium* spp. The bands are numbered. The antisera of tester strains are in the outer wells, antigen in the central well. The well numbers correspond to the anastomosis groups. **A)** Common antigens of *R. solani*; central well contains *R. solani* antigen. **B)** Common antigens shared by *R. solani* and *Ceratobasidium* spp.; central well contains *Ceratobasidium* antigen.

Group 3 was distinguished by bands 9, 10, 11, and 12 (Fig. 3D) and Group 5 by the strongly arcing band 8 (Fig. 3F). The primary difference between AG-1 and AG-4 was band 4 (Fig. 2A). Band 13 was present only in isolates of AG-1 and AG-4; unfortunately, it was not always visible. Isolates of AG-1 and AG-4 appeared serologically similar when antigen concentrations were low. Isolates of *Ceratobasidium* spp. also appeared to be serologically similar to AG-1 (Fig. 2B).

Cross-absorption analysis. Cross absorbing the antiserum of each tester strain with antigens of *Ceratobasidium* spp. removed the common precipitin bands 2, 3, and 4 (Fig. 2B). This clarified relationships between the six anastomosis groups. Isolates of AG-1 and AG-4 appeared identical. One precipitin band was shared by AG-3 and AG-2 types 1 and 2 that was absent in other groups. However, AG-3 formed three other bands that were unique for antigens of AG-3. Isolates in AG-2 types 1 and 2 were identical and formed two distinctive bands; isolates of AG-5 retained one distinctive band.

Cross-absorption tests were performed to isolate the specific antigens unique to each anastomosis group. The antiserum from each tester strain was absorbed with equal concentrations of each of the antigen preparations from the five other tester strains. Each absorbed antiserum then was tested by gel-diffusion against each of the antigen preparations of all six tester strains. One precipitin band remained between the antiserum and antigen of AG-5 and between the antiserum and antigen of AG-2 types 1 and 2. Two bands remained between the antiserum and antigen of AG-3. Anastomosis groups AG-1 and AG-4 had no unique antigens, since their antisera formed no precipitin bands. Thus, through cross-absorption, the antisera of tester strains of AG-2 types 1 and 2, AG-3, and AG-5 each recognized only their specific anastomosis group.

Rapid assay for grouping field isolates. Antiserum from the tester strain AG-3 formed multiple precipitin bands only with field isolates belonging to AG-3. Isolates belonging to AG-1, AG-2 type 1, AG-2 type 2, AG-4, and AG-5 formed one to two precipitin bands (Fig. 1A). Antiserum from tester strain AG-4 formed multiple bands only with AG-4 isolates; isolates from the other five groups formed one to two bands (Fig. 1B). These results permit easy recognition of the serologic grouping of an isolate of *R. solani*, using the simplest method of antigen preparation.

Nature of the antigens. When the antigens of each of the tester strains used for immunization were autoclaved and retested by gel diffusion, they all retained at least three precipitin bands. Surprisingly, these were the common antigens of *R. solani*, that is, bands 2, 3, and 4 and their respective antigen titers were approximately the same as before autoclaving. Of special interest was the persistence of a distinguishing band, possibly band 6 (Fig. 3B, C), for AG-2 types 1 and 2, as well as band 8 (Fig. 3F) for AG-5. After the pronase treatment, followed by autoclaving, these bands disappeared. However, two of the common antigen bands 3 and 4 were still present. We suggest that these common antigens are either unusually stable proteins or nonproteinaceous substances.

DISCUSSION

The results support the premise that each of the anastomosis groups represents a biological species within the taxonomic species *R. solani*. In addition, the presence of three precipitin band systems (Fig. 2A) common to all isolates, regardless of anastomosis group, demonstrates a fundamental similarity among anastomosis groups and supports the present taxonomic species concept of *R. solani*. A caution should be stated that the three precipitin band systems (Fig. 2A) shared by all isolates of *R. solani* might not be useful in identifying *R. solani* from similar fungi. *Ceratobasidium* spp. share many of these common antigens; doubtless, other species of *Thanatephorus* Donk might share bands with *R. solani*. However, differences in band patterns would be apparent when antigens of these fungi are compared with their own antisera rather than with antisera of *R. solani*. Because of genetic variability in *R. solani*, we had expected serologic variability among isolates within anastomosis groups, especially within AG-1 and AG-2. Our serologic techniques did not reflect the diverse cultural characteristics of AG-1 or the discordant patterns of behavior in anastomosis between group 2 isolates reported by Sherwood (16) and Parmeter et al (11). A different experimental approach with antisera formed from isolates of various cultural types within one anastomosis group (for example, AG-1 type 1, 2 and 3 [16]) would be a better approach for examining intragroup diversity by serology.

The serologic results demonstrated the uniformity in AG-2. In our opinion the subgroups AG-2 types 1 and 2 should not be considered as separate groups but as AG-2. Nevertheless, isolates within this group differ in their ability to anastomose. Apparently isolates of AG-2 type 2 have lost the capacity for frequent anastomosis with isolates of AG-2 type 1. Because of this behavior, Parmeter et al (11) suggested the use of three or more tester strains to distinguish isolates of AG-2. The serologic results with AG-2 suggest that the antigens used in this study were not associated directly with the process of anastomosis.

AG-1 and AG-4 were occasionally difficult to separate by gel-diffusion analysis. This was not expected because the cultural characteristics of AG-4 usually are distinguishable from other groups, including group 1. However, it was never difficult to separate AG-1 antigens from AG-4 antigens serologically when they were pre-

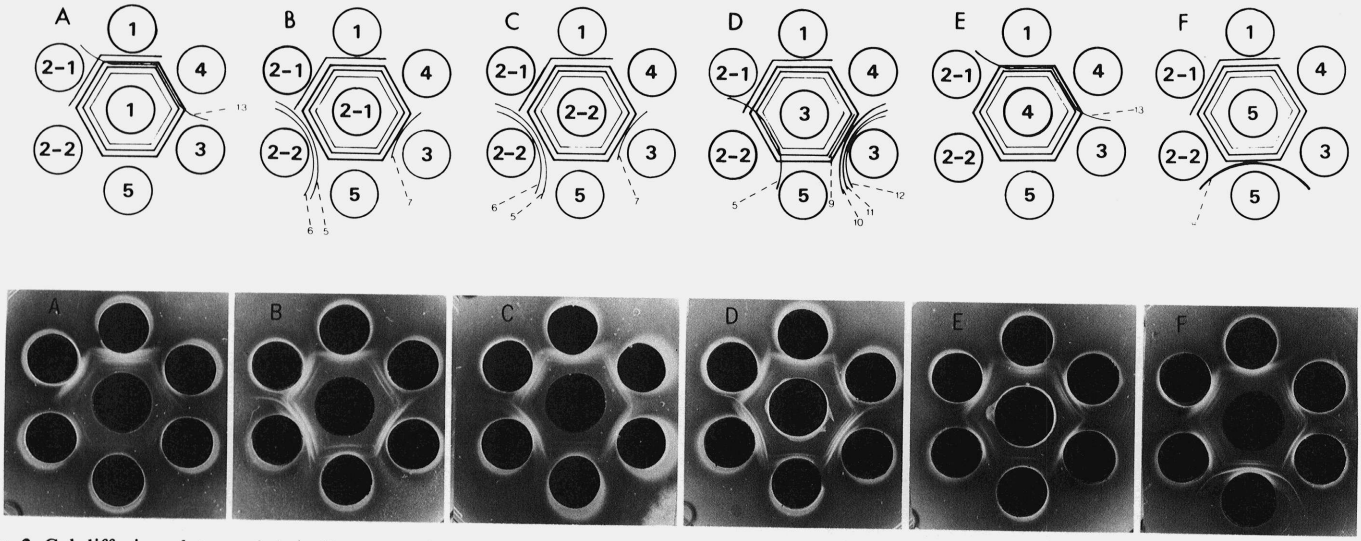

Fig. 3. Gel-diffusion plates and their diagrammatic representations showing precipitin bands formed between antigens and the antisera of six anastomosis groups of *Rhizoctonia solani*. **A)** AG-1. **B)** AG-2 type 1. **C)** AG-2 type 2. **D)** AG-3. **E)** AG-4. **F)** AG-5. Each precipitin band is numbered. Sera of the tester strains are in the outer wells, antigen in the central well. The well numbers correspond to anastomosis groups.

pared by mortar and pestle and tested against AG-4 antiserum with the rapid assay. We believe this difficulty occurred because specific precipitin-band systems for AG-4 are masked when the six test antisera are tested under the conditions of the gel-diffusion experiments. The common antigen bands appear to be superimposed over group-specific bands.

Antisera that react preferentially with the common antigens of *R. solani* appear to be of little value in distinguishing anastomosis groups. The gel patterns of AG-1 and AG-4 vary little from the common precipitin bands (Fig. 3A, E). Therefore, specific antigens may exist that would distinguish AG-1 from AG-4 or show variability between subgroups of AG-2. These problems perhaps could be resolved if the group-specific antigens were isolated and purified.

Many problems arise in the interpretation of serologic reactions when crude antigens are used for immunization. Not all antigens present in a crude antigen mixture necessarily stimulate the formation of antibodies at titers sufficient to develop visible bands in gel-diffusion plates. The ratio of the individual antigens in the mixture influences the titers. The serologic results demonstrate that the antisera of AG-2 types 1 and 2 are not identical and suggest that antigens of AG-2 type 1 preferentially stimulate antibodies for the common antigens rather than the group-specific antigens. Nevertheless, the antigens from the subgroups of AG-2 formed identical precipitin bands when tested against the six test antisera (Fig. 3B,C), proving the homogeneity of AG-2.

The antigenic substances of fungal mycelium are relatively unknown and their antigenicity is notoriously poor. Previous researchers (3,6,14) found that special techniques are necessary to obtain high antibody titers. Probably the most important factor in obtaining high titers is the use of highly concentrated antigens prepared from young mycelium. In *R. solani*, antigens containing at least 10 mg protein per milliliter appear to be required to form titers adequate for gel-diffusion tests. This requirement may be due to the nature of the antigens and the probability that they exist at low concentrations in the saline-soluble protein fraction used for immunization. The antigens of *R. solani* should be characterized so that a better method can be developed to calculate their concentration before injection.

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