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Genetic relationships among isolates of *Rhizoctonia solani* anastomosis group-2 based on isozyme analysis

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Genetic relationships among 14 isolates of *Rhizoctonia solani* anastomosis group AG-2 were studied by evaluating electrophoretic data derived from 11 enzyme systems. All enzymes examined were variable across the isolates studied. The maximum parsimony and unweighted pair-group arithmetic average methods of analysis resulted in similar trees of relationships among isolates. Three closely related groups of isolates, designated groups A, B, and C, were differentiated by isozyme analysis. They were congruent with previous groupings of AG-2 isolates based on studies of anastomosis, morphology, physiology, and DNA/DNA hybridization. Group A consisted of AG-2-1 isolates F56L and 48; group B consisted of AG-2-2 IIIB isolate C330 and AG-2-2 isolates 61D-3, 65L-2, and Rhs36; group C consisted of AG-2-2 IV isolate RI-64 and AG-2-2 isolates H3-77, H500, 86-42-4, 86-62-1, 86-72-7, 133-A-4, and 454. Group A was less associated with group B and C, with a coefficient of association of 0.18, compared with 0.27 for that between the latter two groups. Most isolates of group C were obtained from sugarbeets, while those of group B had a broader host range and group A isolates are of unknown origin.

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Les relations génétiques entre 14 isolats de *Rhizoctonia solani* du groupe anastomosique AG-2 ont été étudiées par analyses électrophorétiques de 11 systèmes enzymatiques. Parmi tous les isolats, une variabilité a été observée chez toutes les enzymes étudiées. Les méthodes d'analyse de "parcimonie maximale" et de groupement selon la moyenne arithmétique ont révélé des arbres relationnels similaires. Trois groupes d'isolats très reliés, désignés groupes A,B, et C, ont pu être différenciés par l'analyse des isozymes. Ceux-ci concordent avec les regroupements des isolats d'AG-2 déjà établis au moyen des études d'anastomose, de morphologie, de physiologie et d'hybridation ADN/ADN. Le groupe A est composé des isolats F56L et 48 du groupe AG-2-1; le groupe B, de l'isolat C330 du sous-groupe AG-2-2 IIIB et des isolats 61D-3, 65L-2, et Rhs 36 du groupe AG-2-2; tandis que le groupe C est constitué de l'isolat RI-64 du sous-groupe AG-2-IV et des isolats H3-77, H500, 86-42-4, 86-62-1, 86-72-7, 133-A-4, et 454 du groupe AG-2-2. Le groupe A a été moins associé aux groupes B et C avec un coefficient d'association de 0,18 par rapport à 0,27 entre les derniers groupes. La plupart des isolats du groupe C ont été isolés de la betterave, ceux du groupe B ont une gamme d'hôtes plus étendue, et les isolats du groupe A sont d'origine inconnue.

Rhizoctonia solani Kühn [teleomorph *Thanatephorus cucumeris* (Frank) Donk], a common Basidiomycete plant pathogen with a broad host range, causes a variety of plant diseases, including pre- and postemergence damping-off, crown and root rot, and foliage blights (Sinclair & Backman 1989). The anamorphic stage produces sclerotia but no asexual spores and is composed of multinucleate hyphal cells with the number of nuclei per cell varying among isolates. The teleomorphic state, *T. cucumeris*, has been confirmed for all recognized groups of *R. solani* (Carling et al. 1987, Ogoshi 1987), but this stage is rarely seen in nature. Obligate heterothallism or homothallism have been noted for some isolates (Adams 1988). As a taxonomic entity, *T. cucumeris* is one of the most diverse and complex fungi ever considered a single species. A system using anastomosis group (AG), based on hyphal fusion between and among various isolates, is widely accepted as the basis for classifying subspecific groups of this fungus (Ogoshi 1976, 1987; Parmeter et al. 1969, Schultz 1936). Ogoshi (1984, 1987) introduced the concept of intraspecific group (ISG) to better understand anastomosis grouping. Ten AGs or 14 ISGs of *R. solani* have been recognized (Carling et al. 1987, Liu 1990, Ogoshi 1987). Rhizoctonia diseases of soybeans [*Glycine max* (L.) Merr.] are caused mainly by members of AG-4 (Anderson 1982). Two isolates highly virulent to soybeans, 61D-3 and 65L-2, obtained from Illinois field-grown soybeans, were identified as members of AG-2-2 (Liu 1990, Liu & Sinclair 1991). AG-2 has two subgroups (AG-2-1 and AG-2-2) containing important plant pathogens (Anderson 1982); however, their relationship is poorly understood.

Enzyme electrophoresis has been used in genetic studies of higher organisms, including plant pathogenic fungi (Micales et. al. 1986, Newton 1987). Electrophoretic banding patterns of isozymes are usually predictable, based on their genetic background. Isozyme analysis has a potential for resolving relationships among imperfect fungi (Bosland & Williams 1987). For fungal isolates with few or no readily distinguishable morphological features, such as *R. solani*, biochemical probes may provide markers useful for classification. Previous electrophoretic work on

Table 1. Isolates of *Rhizoctonia solani* anastomosis group AG-2 and one isolate of AG-1 as outgroup used for isozyme analysis in 11 enzyme systems

ISG[y]	Isolate no.	Origin	Source[z]/host
AG-1	7317	Java	D.E. Carling/ unknown
AG-2-1	F56L	Alaska	D.E. Carling/ unknown
AG-2-1	48	Australia	N.A. Anderson/ unknown
AG-2-2	H3-77	Minnesota	N.A. Anderson/ carrot
AG-2-2	H500	Ohio	L.J. Herr/ sugarbeets
AG-2-2	Rhs36	Georgia	D.R. Sumner/ maize
AG-2-2	61D-3	Illinois	Z. Liu/ soybeans
AG-2-2	65L-2	Illinois	Z. Liu/ soybeans
AG-2-2	86-42-4	Minnesota	C. E. Windels/ sugarbeets
AG-2-2	86-62-1	North Dakota	C.E. Windels/ sugarbeets
AG-2-2	86-72-7	Minnesota	C.E. Windels/ sugarbeets
AG-2-2	133-A-4	Minnesota	C.E. Windels/ sugarbeets
AG-2-2	454	California	E.E. Butler/ sugarbeets
AG-2-2 IIIB	C330	Japan	A. Ogoshi/ mat rush
AG-2-2 IV	RI-64	Japan	A. Ogoshi/ sugarbeets

[y]ISG: intraspecific group based on Ogoshi's system (1987).
[z]Address: N.A. Anderson, Dept. of Plant Pathology, Univ. of Minnesota, St. Paul, 55108, USA; E.E. Butler, Dept. of Plant Pathology, Univ. of California, Davis, 95616, USA; D.E. Carling, Univ. of Alaska, 533 E. Fireweed, Palmer, 99645, USA; L.J. Herr, Dept. of Plant Pathology, Ohio State Univ., Wooster, 44691, USA; Z.L. Liu, Dept. of Plant Pathology, Univ. of Illinois, Urbana, IL 61801; A. Ogoshi, Hokkaido Univ., N9 W9 Juta-Ku, Sapporo 060, Japan; D.R. Sumner, Coastal Plain Experiment Station, P.O. Box 748, Tifton, GA 31793, USA; and C.E. Windels, North West Experiment Station, Crookston, MN 56716, USA.

R. solani used either a protein stain (Clare et al. 1968, Reynolds et al. 1983) or involved only one enzyme system (Matsuyama et al. 1978) and did not address genetic or phylogenetic relationships. Laroche et al. (1989, 1990) concluded from preliminary studies using isozyme analysis that the present grouping of two AGs of *R. solani* based on anastomosis was valid and indicative of genetic relatedness.

The objective of this study was to compare relationships of representative isolates of different AGs and ISGs based upon anastomosis and pathogenicity groupings with those determined by isozyme analysis. We focused on the relationships within AG-2. These results can serve as a model for further studies to propose a phylogeny for this fungus. Previous results were reported elsewhere (Liu et al. 1989).

Materials and methods

Isolate collection and culture maintenance. Fourteen isolates of *R. solani* representing different subgroups of AG-2 and one AG-1 isolate as outgroup from Australia, Indonesia, Japan, and the U.S. were studied (Table 1). Each isolate was maintained on Difco potato-dextrose agar (PDA) plates and slants at 4 and 20°C, respectively. Initially, each isolate was cultured in 9-cm PDA culture plates at its optimum temperature for growth (range 20-30°C). Each culture was transferred via hyphal tip every other day until fast, uniform growth was obtained. No culture with sectoring was used.

Enzyme extraction. For each isolate, three agar plugs containing fresh hyphal tips were excised from 2-day-old PDA plates and transferred to 50 mL of potato-dextrose broth (potato infusion 200 g, Bacto dextrose 20 g/L) in a 250 mL erlenmeyer flask. Each flask was incubated on a rotary shaker (120 rpm) for 6 days at 25 ± 1°C. One mycelial mat (two for slow-growing cultures) was harvested by decanting off the broth, then

Table 2. Enzymes, enzyme commission numbers and buffer systems used to evaluate relationships of isolates of *Rhizoctonia solani*

Enzyme	Abbreviation	E.C. no.	Buffer[z]
Acid phosphatase	ACP	3.1.3.2	1
Aconitase	ACO	4.7.1.3	2
Esterase	EST	3.1.1.1	3
Glutathione reductase	GSR	1.6.4.2	1
Hexose kinase	HXK	2.7.1.1	1
Isocitric dehydrogenase	IDH	1.1.1.42	1
Leucine amino peptidase	LAP	3.4.11.1	3
Malate dehydrogenase	MDH	1.1.1.37	2
Phosphoglucomutase	PGM	2.7.5.1	3
Phosphoglucoisomerase	PGI	5.3.1.9	3
6-Phosphogluconate dehydrogenase	6-PGD	1.1.1.44	1

[z] 1 = Tris-citrate (trizma base 0.223 M, citric acid 0.086 M adjust with 1.0 N NaOH, pH 7.5) (Soltis et al. 1983);
2 = Citrate-morpholine [citric acid (anhydrous) 0.04 M, adjust to pH 6.5 with N-(3-aminopropyl)-morpholine for electrode buffer, pH 6.0 for gel buffer] (Clayton & Tretiak 1972; Nickrent 1986);
3 = Tris-EDTA-borate (tris 0.5 M, NaEDTA 0.02 M, boric acid 0.65 M, adjust to pH 8.1 with 1.0 N NaOH) (Ridgway et al. 1970).

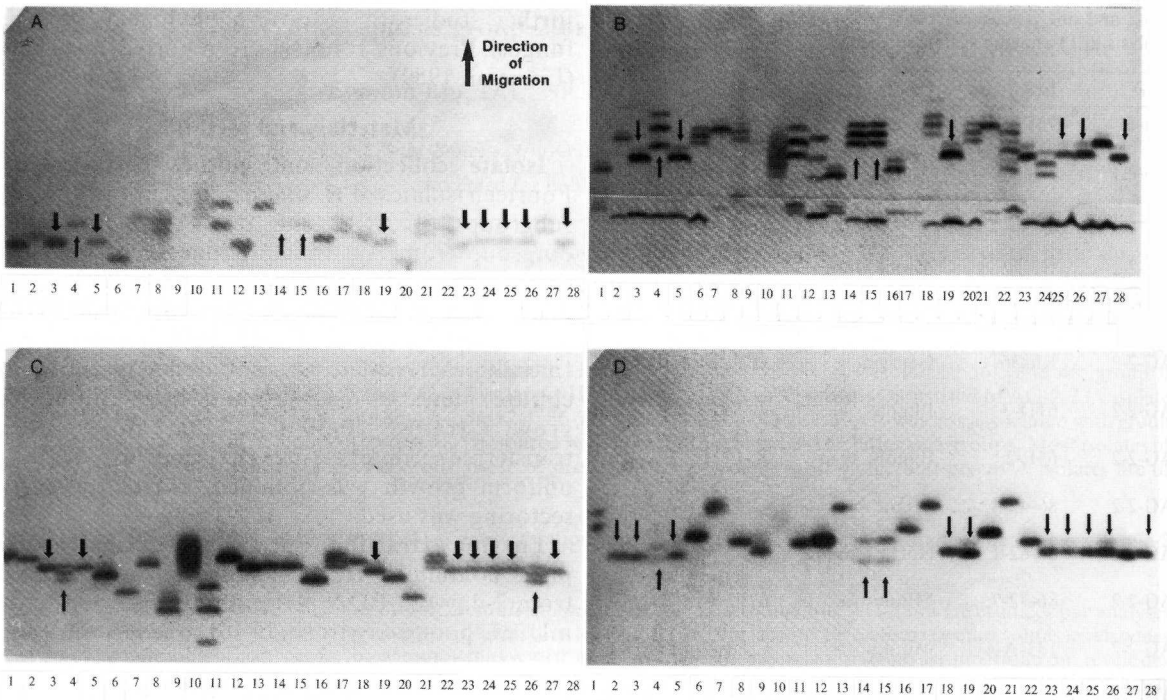

Figure 1. Selected starch gels of four isozyme systems: ACO (**A**), MDH (**B**), PGI (**C**), and PGM (**D**), showing shared bands and variations among 28 isolates of 10 AGs or 12 ISGs of *Rhizoctonia solani*. Upward pointing arrows indicate shared bands of the members of the group B (4 = ISG AG-2-2 IIIVB C330, 14 = AG-2-2 61D-3, 15 = AG-2-2 65L-2, and 27 = AG-2-2 Rhs36); downward pointing arrows indicate shared bands for the members of the group C (3 = AG-2-2 454, 5 = ISG AG-2-2 IV R1-64, 19 = AG-2-2 H3-77, 23 = AG-2-2 133-A-4, 24 = AG-2-2 86-42-4, 25 = AG-2-2 86-62-1, 26 = AG-2-2 86-72-7, and 28 = AG-2-2 H500); and unmarked shared and varied banding for the group A isolates (2 = AG-2-1 F56-L, 18 = AG-2-1 48). Other lanes refer to isolates of AGs not discussed in this paper. Direction of migration of bands is from bottom to top (arrow).

press-rinsing it twice in an enzyme extraction buffer. The extraction buffer (pH 7.0) was composed of 0.1 M Tris, 10.0 mM KCl, 0.1 mM $MgCl_2$, 1.0 mM ethylene diamine tetra-ascorbic acid, 14.0 mM β-mercaptoethanol, 0.1 mM ascorbic acid, and polyvinylpyrrolidone (0.2 g/mL of buffer). Mycelial mats for each isolate were ground separately in 1.5 mL extraction buffer using a glass homogenizer. The homogenate was then transferred to a precooled 10 mL beaker submerged in ice water, and sonicated (Branson 450 Sonifier) three times for 10 seconds each at 10-second intervals. Sonicated preparations were centrifuged at 13 000 rpm for 30 min at 4° C and the supernatant transferred to 1.5 mL microfuge tubes. All extracts were used either fresh or within 24 h after being frozen at –80° C.

Starch gel electrophoresis. Horizontal starch gels (Sigma Chemical Co., St. Louis, Mo) were electrophoresed as described by Micales et al. (1986), and stained according to Soltis et al. (1983). Eleven resolved enzymes of 29 tested and the three buffer systems used are shown (Table 2). Repeatable banding patterns were obtained for all isolates tested and at least two complete gel sets were run for each isolate.

Data analysis. Due to the lack of information concerning the genetics of isozyme variation for this fungus, a conservative approach was taken. In most cases, the term band was used instead of allele. For each enzyme, all the bands that appeared on a gel were measured for their relative mobilities to the anode with the most common band designated as 100. Each single band was considered as an independent character (Buth 1984). Bands observed as discrete (binary) characters for each enzyme system were compiled into a matrix. The data codes for the matrix were: band present [1], band absent [0]], and missing data [9]. Numerical cladistic analyses were performed with the aid of the PAUP program (Swofford 1985), which seeks trees that are optimal under the criterion of maximum parsimony. Algorithms for finding most-parsimonious trees minimize the amount of evolutionary change needed to explain a given set of data. The assumptions of parallelism, conver-

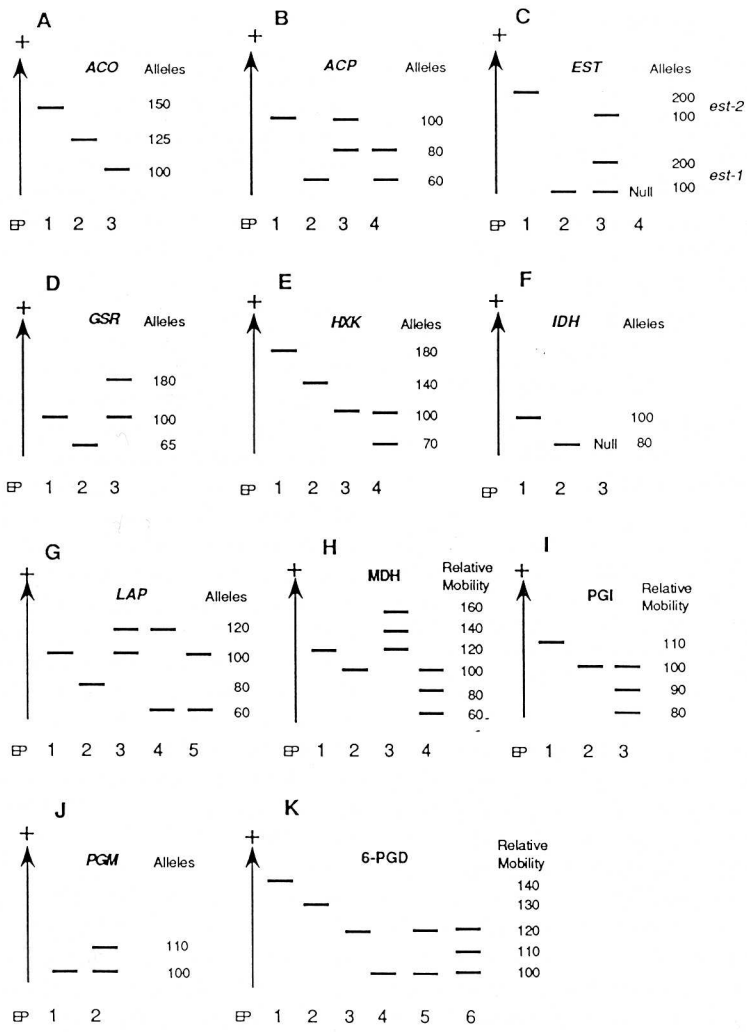

Figure 2. Electrophoretic phenotypes (EP) observed for 11 enzyme systems (A-K) for 14 AG-2 isolates of *Rhizoctonia solani*. The numbers assigned are relative mobilities to the anode compared with the most common band (100). The banding patterns of malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), and 6-phosphogluconate dehydrogenase (6-PGD), which were not interpretable, were treated as discrete banding characters only.

gence, and reversal are invoked only when other less ad hoc explanations for the sharing of similar character states are insufficient due to conflicts among characters. The method has been used for analyzing morphological (Wiley 1981) and molecular (Swofford & Olsen 1990) data. In this study, when multiple, equal-parsimonious trees were found, majority-rule consensus trees were generated. The topologies shown were those supported by greater than half of the equally-parsimonious trees. Outgroup analyses were used for discrete characters.

Based on the isozyme banding, the coefficients of association were calculated using the formula a/a+b, where a is the number of shared bands and b is the number of unshared bands between isolates. Unweighted pair-group arithmetic average (UPGMA) cluster analysis then was used to show relationships based upon the degree of associations (Ferguson 1980, Sneath & Sokal 1973).

Results

Enzyme variations of *R. solani*. Eleven of 29 enzymes tested were polymorphic for 28 isolates representing 10 AGs or 12 ISGs of *R. solani*. There was a diverse variation of banding patterns among all isolates tested (Fig. 1). However, bands shared by isolates in the same group were also common.

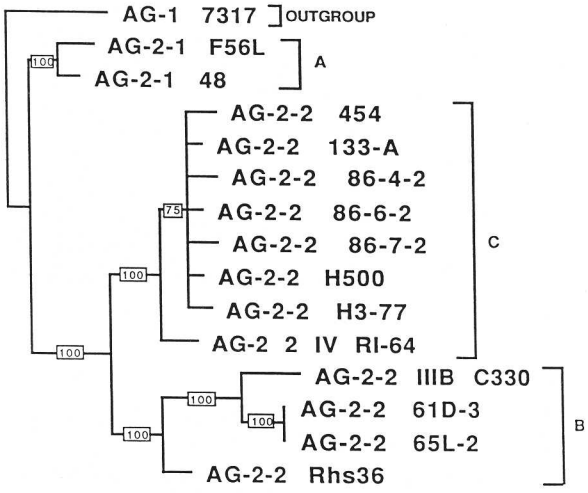

Figure 3. Majority rules consensus phylogram (103 steps; consistency index = 0.553) of four equally parsimonious trees (96 steps each; consistency index = 0.594) derived from isozyme bands showing genetic relationships among 14 AG-2 isolates of *Rhizoctonia solani*, using AG-1 as the outgroup. Numbers within boxes at several nodes indicate the percentage of the four trees supporting this topology.

Using AG-1 as an outgroup, isolates from different AGs or ISGs tended to cluster together with variations. The ISGs representing AG-2 appeared to be monophyletic. Multiple band phenotypes were present in all AG-2 isolates (Fig. 2) but were not confined to any one isolate.

The banding patterns of MDH, PGI, and 6-PGD were not interpretable for the possible genetic base, but were treated as banding characteristics. All other enzymes except EST were presumably coded for a single locus. Enzymes ACO, ACP, and GSR had three bands at various relative mobilities and were considered as three alleles, HXK and LAP had four bands (alleles), and IDH and PGM had two bands (alleles) each. Enzyme EST showed four bands coding for two loci each having two alleles. In our system, no detectable bands showed on the gels for isolates AG-2-1 F56L and AG-2-1 48; and AG-2-2 IIIB C330, AG-2-2 61D-3, and AG-2-2 65L-2 for EST and IDH, respectively. In contrast, various banding patterns for all other AG-2 isolates showed for these two enzymes on the same gels. Therefore, the isolates without the respective banding patterns were assumed to have a homozygous null allele, respectively.

Relationships among 14 isolates of AG-2. Using AG-1 as an outgroup, both heuristic and branch and bound analyses resulted in four equally-parsimonious cladograms of 96 steps each. The majority rule consensus phylogram for AG-2 isolates is shown (Fig. 3). For the 14 AG-2 isolates, three clusters resulted: group A (ISG AG-2-1 isolates F56L and 48), group B (ISG AG-2-2 IIIB C330 and AG-2-2 isolates 61D-3, 65L-2, and Rhs 36), and group C (ISG AG-2-2 IV RI-64 and the remaining seven AG-2-2 isolates) (Table 1). Relationships among the eight isolates within cluster C, not fully resolved, are shown as a polychotomy (Fig. 3).

In the phenetic approach, the coefficients of association for the 14 AG-2 isolates were calculated (Table 3). A UPGMA phenogram constructed from the degree of associations showed relationships similar to the previous cladogram (Fig. 4). In this phenogram, three clusters were retained with

Table 3. Coefficients of association for 14 AG-2 isolates of *Rhizoctonia solani*

Isolate No/.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.00													
2	0.63	0.00												
3	0.13	0.16	0.00											
4	0.09	0.08	0.53	0.00										
5	0.22	0.16	0.23	0.24	0.00									
6	0.17	0.29	0.14	0.11	0.44	0.00								
7	0.17	0.29	0.14	0.11	0.48	0.89	0.00							
8	0.08	0.07	0.68	0.63	0.26	0.13	0.13	0.00						
9	0.09	0.08	0.56	0.59	0.25	0.20	0.21	0.67	0.00					
10	0.08	0.07	0.50	0.81	0.19	0.14	0.14	0.60	0.75	0.00				
11	0.09	0.07	0.58	0.93	0.23	0.10	0.10	0.68	0.65	0.88	0.00			
12	0.12	0.19	0.43	0.52	0.38	0.30	0.30	0.35	0.48	0.43	0.50	0.00		
13	0.23	0.29	0.15	0.19	0.48	0.57	0.62	0.18	0.25	0.23	0.19	0.52	0.00	
14	0.04	0.07	0.53	0.65	0.15	0.15	0.11	0.48	0.42	0.61	0.71	0.33	0.14	0.00

Isolate number: 1 = AG-2-1 F56L, 2 = AG-2-1 48, 3 = AG-2-2 H3-77, 4 = AG-2-2 H500, 5 = AG-2-2, Rhs36, 6 = AG-2-2 61D-3, 7 = AG-2-2 65L-2, 8 = AG-2-2 86-42-2, 9 = AG-2-2 86-62-1, 10 = AG-2-2 86-72-7, 11 = AG-2-2 133-A-4, 12 = AG-2-2 454, 13 = AG-2-2 IIIB C330, 14 = AG-2-2 IV RI-64.

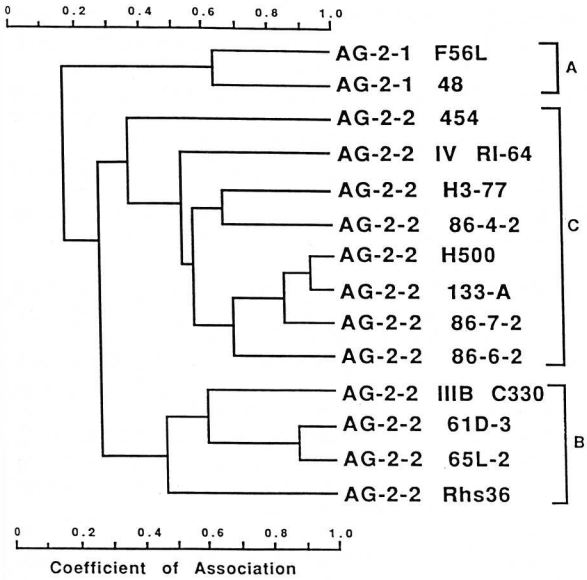

Figure 4. Phenogram of unweighted pair-group arithmetic average derived from the degree of association showing overall similarity for 14 AG-2 isolates of *Rhizoctonia solani*.

the group A cluster, being most dissimilar, with a coefficient of association of 0.18 to group B and C. The topology of cluster for group B remained the same, while that for group C, comprising eight AG-2-2 isolates, varied in the placement of several isolates relative to the cladogram.

Discussion

Rhizoctonia solani AG-2 was reported to have two subgroups, AG-2-1 and AG-2-2, based on the relative frequency of hyphal fusion, cultural characteristics, and thiamine dependence (Ogoshi 1976, Ogoshi & Ui 1979, Watanabe & Matsuda 1966). Further studies divided AG-2-2 into two ecological types, AG-2-2 IIIB and AG-2-2 IV, based on cultural characteristics and pathogenicity to mat rush (*Juncus effusus* L. var. *decipiens*) and sugarbeets (*Beta vulgaris* L.), respectively (Ogoshi 1987, Watanabe & Matsuda 1966). However, these two subgroups were not distinguishable by hyphal anastomosis. None of the aforementioned AG-2 subgroups has been differentiated serologically or by electrophoretic studies (Matsuyama et al. 1978, Reynolds et al. 1983). Similarly, the DNA/DNA hybridization and restriction fragment length polymorphism of ribosomal DNA studies did not differentiate AGs or ISGs among isolates of *R. solani* (Vilgalys 1988, Vilgalys & Gonzalez 1990).

By isozyme analysis, *R. solani* showed high levels of genetic variation, similar to isozyme studies of other fungi (Bonde et al. 1984, May & Royse 1988, Newton 1987, Royse & May 1982). High levels of genetic variability was reported among members of the Basidiomycetes and species of *Phytophthora* in their sexual rather than asexual state (Burdon & Roelfs 1985, Todey et al. 1985). *R. solani* may have the same trend (Adams 1988, Anderson 1982). Using isozyme analysis we found sufficient variation within the asexual state of this fungus to allow inter-isolate and inter-group comparisons. Cladistic and phenetic analyses derived from enzyme electrophoretic data showed common trends in the relationship among the isolates of *R. solani* studied. The 14 isolates of AG-2 studied were differentiated as three groups, related to ISG AG-2-1, ISG AG-2-2 IIIB, and ISG AG-2-2 IV, respectively. This grouping was congruent with those determined by examination of anastomosis, morphology, physiology, behavior, and pathogenicity tests (Ogoshi 1987, Ogoshi & Ui 1979, Watanabe & Matsuda 1966). These results suggested that groups B and C are more closely related to each other than either is to group A. This relationship is also supported by previous DNA homology and DNA/DNA hybridization studies (Kuninaga 1986, Kuninaga & Yokosawa 1982, Vilgalys 1988).

Our two Illinois isolates, 61D-3 and 65L-2, previously classified as AG-2-2 by anastomosis grouping (Liu & Sinclair 1991), were shown to be related to ISG AG-2-2 IIIB in group B. Group B also contained isolate Rhs 36, an isolate from Georgia that induces crown and brace root rot in maize (*Zea mays* L.) (Sumner & Bell 1982), which was previously placed in AG-2-2. The seven AG-2-2 isolates from North America appeared related to ISG AG-2-2 IV from Japan in group C. Most isolates of group C, except one, are primarily pathogens of sugarbeets, while members of group B have a broad host range. AG-2-2 IIIB type was reported only in Japan and designated as a separated ISG (Ogoshi 1987). Our results suggest that some U.S. isolates of AG-2-2 are related to this type. The ISG AG-2-2 IIIB type could be a distinct group of genetic relatedness in addition to ISG AG-2-2 IV. The separation of the three subgroups in AG-2 by isozyme study provides information for the understanding and further studies on the relationship among AG-2 isolates as well as the entire species.

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