Grouping of isolates in AG 2 of *Rhizoctonia solani* by total cellular fatty acid analysis

Masaru Matsumoto[1] and Nobuaki Matsuyama[2]

[1] Institute of Tropical Agriculture, Kyushu University, 6–10–1, Hakozaki, Fukuoka 812–8581, Japan
[2] Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University, 6–10–1, Hakozaki, Fukuoka 812–8581, Japan

Total-cellular fatty acid compositions of 34 isolates of *Rhizoctonia solani* belonging to intraspecific groups (ISGs) of anastomosis group (AG) 2, i.e., AG 2-1, AG 2-2 IIIB (mat rush), AG 2-2 IV (sugar beet), AG 2-2 LP (turfgrass), and AG 2-3 (soybean), were compared. The major fatty acids identified were palmitic, stearic, and oleic acids. Principal component analysis based on the percentage composition of total cellular fatty acids revealed consistently low variability among isolates of a single ISG of AG 2. Average linkage cluster analysis showed that isolates obtained from turfgrass representing a newly proposed group, AG 2-2 LP, were differentiated from other AG 2 ISGs. Isolates of another newly proposed group AG 2-3, from diseased soybean were also closely related to AG 2-1 and AG 2-2 IIIB but distinguishable from the AG 2-1 and AG 2-2 LP isolates by the average linkage cluster analysis. These results suggested that the percentage composition of total-cellular fatty acids is a distinct characteristic for the five ISGs belonging to AG 2, and fatty acid analysis is useful for the differentiation and characterization of these ISGs of AG 2 in *R. solani*.

Key Words——fatty acid analysis; PCR-RFLP analysis; *Rhizoctonia solani* AG 2.

The phytopathogenic fungus *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a complex species varying in morphological, pathological, and physiological characteristics among isolates (Adams, 1988). Hyphal anastomosis in *R. solani* was first described by Matsumoto (1921). Currently, 11 anastomosis groups (AGs) are recognized (Carling et al., 1987; Ogoshi, 1987; Ogoshi et al., 1990), and 1 group (AG 2) is further divided into intraspecific groups (ISGs) based on anastomosis frequency (Ogoshi, 1976) and thiamine requirement (Sherwood, 1969; Ogoshi and Ui, 1979).

AG 2 was first subdivided into two ISGs, AG 2-2 IIIB and AG 2-2 IV, based on pathogenicity and cultural characteristics (Watanabe and Matsuda, 1966). *Rhizoctonia solani* AG 2-2 IIIB is a causal agent of sheath blight of cultivated mat rush (*Juncus effusus* L. var. *decipiens* Buch.) and false sheath blight of rice (*Oryza sativa* L.), whereas AG 2-2 IV causes root rot and leaf blight of sugar beet (*Beta vulgaris* L.).

Isolates of AG 2-2, as a whole, attack a wide range of crops. AG 2-2 also causes brown patch of St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze) (Hurd and Grisham, 1983; Haygood and Martin, 1990; Burpee and Martin, 1992), large patch of turfgrass (*Zoysia japonica* Steud.), and root rot of soybean (*Glycine max* Merr.) (Liu and Sinclair, 1991). Recently, new populations in AG 2, designated as AG 2-2 LP and AG 2-3, have been reported in Japan. Isolates of AG 2-2 LP are a causal agent of large patch disease of Japanese

turfgrass (*Z. japonica*) (Hyakumachi et al., 1998), and isolates of AG 2-3 are a causal agent of foliar blight disease of soybean (Naito and Kanematsu, 1994).

The ISGs within AG 2-2 cannot be distinguished by anastomosis (Ogoshi, 1976), thiamine requirement (Ogoshi and Ui, 1979), DNA base composition (Kuninaga and Yokosawa, 1980), or ribosomal DNA restriction fragment length polymorphisms (RFLPs) (Vilgalys and Gonzalez, 1990), but are distinguishable by DNA base sequence homology (Kuninaga and Yokosawa, 1982), zymogram analysis (Matsuyama et al., 1978; Liu et al., 1990), restriction analysis of PCR-amplified ribosomal DNA (rDNA) (Liu and Sinclair, 1992), and whole cell fatty acid analysis (Stevens Johnk and Jones, 1992).

The aim of this study is to assess the genetic diversity of AG 2 ISGs consisting of AG 2-1, AG 2-2 IIIB, AG 2-2 IV, and two newly recognized populations in Japan, AG 2-2 LP and AG 2-3, by comparing total cellular fatty acid compositions. Fatty acid analysis has successfully characterized closely related groups of phytopathogenic bacteria. Cultural appearance and thiamine requirement of isolates within ISGs were also examined. Preliminary results of these analyses have been published (Matsumoto et al., 1996).

Materials and Methods

Fungal isolates The 34 isolates of *R. solani* AG 2 used in this study are listed in Table 1. They include seven isolates each of AG 2-1, AG 2-2 IIIB, AG 2-2 IV, and AG 2-2

Table 1.   Isolates of *Rhizoctonia solani* AG 2 tested.

Isolate	AG	Origin	Source[a]
PS-4	2-1	Pea	ATCC 76124
SH-3	2-1	Soil	IFO 30940
F-15	2-1	Flax	IFO 30941
TG-1	2-1	Tulip	AKU
R1-2-4	2-1	Barley	MAFF 305203
BO-1	2-1	Cabbage	MAFF 305237
HV-1	2-1	Barley	MAFF 305238
C-96	2-2 IIIB	Mat rush	ATCC 76124
C-100	2-2 IIIB	Mat rush	MAFF
C-116	2-2 IIIB	Mat rush	MAFF
C-321	2-2 IIIB	Mat rush	IFO 30943
C-328	2-2 IIIB	Mat rush	IFO 30944
C-335	2-2 IIIB	Mat rush	MAFF
C-354	2-2 IIIB	Mat rush	AKU
RI-64	2-2 IV	Sugar beet	MAFF
B-70	2-2 IV	Sugar beet	AHU
Pf-28	2-2 IV	Sugar beet	MAFF
Rh-46	2-2 IV	Sugar beet	MAFF
Rh 509-S-1	2-2 IV	Sugar beet	IFO 30796
BV-28	2-2 IV	Sugar beet	AKU
C-34	2-2 IV	Sugar beet	MAFF 305245
48R	2-2 LP	Zoysia grass	AGU
G1	2-2 LP	Zoysia grass	AGU
G3	2-2 LP	Zoysia grass	AGU
K1-9	2-2 LP	Zoysia grass	AGU
No.87	2-2 LP	Zoysia grass	AGU
RGR-38	2-2 LP	Bermuda grass	AGU
SLP 3-1	2-2 LP	St. Augustine grass	AGU
R-1	2-3	Soybean	MAFF 235446
R-6	2-3	Soybean	MAFF 235448
H4-38-S-1	2-3	Soybean	MAFF 235449
H5-307	2-3	Soybean	MAFF 235450
H5-354	2-3	Soybean	MAFF 235452
H5-358	2-3	Soybean	MAFF 235453

a) AHU: Faculty of Agriculture, Hokkaido University, Hokkaido, Japan.
AKU: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
IFO: Institute for Fermentation, Osaka, Japan.
ATCC: American Type Culture Collection, USA.
MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan.
AGU: Faculty of Agriculture, Gifu University, Gifu, Japan.

LP and six isolates of AG 2-3. All isolates were maintained on slants of potato-dextrose agar (Funakoshi Chem.) at 25°C in the dark.

Thiamine requirement Thiamine requirement of the 34 isolates was determined as previously described (Naito and Kanematsu, 1994). The basal medium (glucose asparagine medium (GA)) consisted of 10 g of D-glucose, 2 g of L-asparagine, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of KH_2PO_4, 0.2 mg of $Fe(NO_3)_3 \cdot 9H_2O$, 0.2 mg of $ZnSO_4 \cdot$

$7H_2O$, and 0.1 mg of $MnSO_4 \cdot 7H_2O$ in 1 L of distilled deionized water. Thiamine hydrochloride (final conc., 3.4 mg/L) was added after autoclaving. Inoculum was grown on the basal agar medium (GA with 15 g/L) without thiamine. Inoculated flasks were incubated in the dark at 28°C for 10 d, then mycelial mats were removed, lyophilized, and weighed. The experiment was repeated twice. Isolate variability in mycelial dry weight was estimated using the statistical software SYSTAT (SYSTAT, Inc).

Total cellular fatty acid analysis Total cellular fatty acid was analyzed according to Matsumoto et al. (1997) as previously reported. Fatty acids extracted from 20 to 30 mg of lyophilized mycelia grown in the liquid nutrient-broth medium at 25°C for 7 d were methylated with 0.5 ml of 5% (v/v) HCl-methanol at 100°C for 3 h in sealed glass tubes. The resultant fatty acid methyl esters (FAMEs) were cooled to room temperature, diluted with 1 ml of distilled water, and extracted with 2 ml of *n*-hexane by shaking. The solvent phase was washed with an equal volume of distilled water to remove HCl and dehydrated by mixing with 0.5 mg of anhydrous sodium sulfate. The solvent was evaporated under nitrogen stream. Samples were stored at $-20°C$.

Samples were analyzed using a gas liquid chromatograph (Shimadzu GC17A) equipped with a flame ionization detector and a 0.25 mm × 50 m HR-SS-10 capillary column. The column and injection temperatures were maintained at 180°C and 250°C, respectively. The pressure of nitrogen gas was 95 kpa. FAMEs were identified by comparing their retention times with those of authentic materials. Peak area was also calculated automatically and expressed as a percentage of total fatty acids detected. Triplicates determinations were made for each isolate. Average values of fatty acid composition were used to differentiate the isolates of ISGs of AG 2. For each isolate, a data record was constructed of percentages of each fatty acid.

Data evaluation Data for five ISGs of *R. solani* AG 2 were presented as mean percentages and standard deviations (SDs) of fatty acids from three replicated samples. Variability among isolates within a single ISG was assessed with respect to both individual and overall fatty acid compositions by the Walter-Duncan *K*-ratio *t*-test ($K=100$, $t=0.05$). In addition, principal component analysis (SYSTAT) was used to determine the amount of variability among isolates. Relatedness between ISGs was also assessed by the average linkage cluster analysis procedure using the SYSTAT software.

Results

Thiamine requirement Isolates of ISGs representing AG 2-2 IIIB, 2-2 IV, 2-2 LP and 2-3 were thiamine auxotrophic, while isolates of AG 2-1 were thiamine autotrophic. Mycelial dry weight (mg) of AG 2-2 ISGs increased significantly ($P<0.001$) in thiamine-amended GA (Table 2). Thiamine amendment did not influence the growth of the isolates of AG 2-1 (Table 2).

Fatty acid analysis Nine fatty acids (myristic ($C14_0$),

pentadecanoic (C15$_0$), palmitc (C16$_0$), palmitoleic (C16$_1$), heptadecanoic (C17$_0$), 9-heptadecenoic (C17$_1$), stearic (C18$_0$), oleic (C18$_1$), and linoleic (C18$_2$) acids) were detected from the 34 isolates of *R. solani* AG 2. Palmitic, oleic, and linoleic acids predominated in all isolates tested (Table 3). The most predominant was linoleic acid, which comprised 76.63% of the total cellular fatty acid on the average; and oleic acid was second with an average of 10.64% in the AG 2 ISGs.

Principal component analysis showed close similarity in the percentage compositions of fatty acid among isolates within a single ISG of AG 2, but divergence among ISGs (Fig. 1.). The top three (palmitic, stearic, and oleic acids) of the nine components (principal components 1, 2, and 3) of this analysis accounted for 35.6% of the variation in the data. Principal components 1, 2, and 3 accounted for 39.6%, 23.7% and 12.5% of the variability, respectively.

The total cellular fatty acid compositions of 34 isolates in AG 2 were compared by average linkage cluster analysis (Fig. 2.). The dendrogram showed that the isolates within a single ISG of AG 2 were clustered within a Euclidean distance of less than 1.2. Isolates of AG 2-1 and AG 2-2 IIIB were closely clustered with a Euclidean distance of 1.5. They were further closely related to isolates of AG 2-3, with a Euclidean distance of 2.0. AG 2-2 IV was separated from AG 2-1, AG 2-2 IIIB, and AG 2-

3, with a Euclidean distance of 3.2. AG 2-2 LP was distant from other ISGs of AG 2, with a Euclidean distance of 5.0 in the dendrogram.
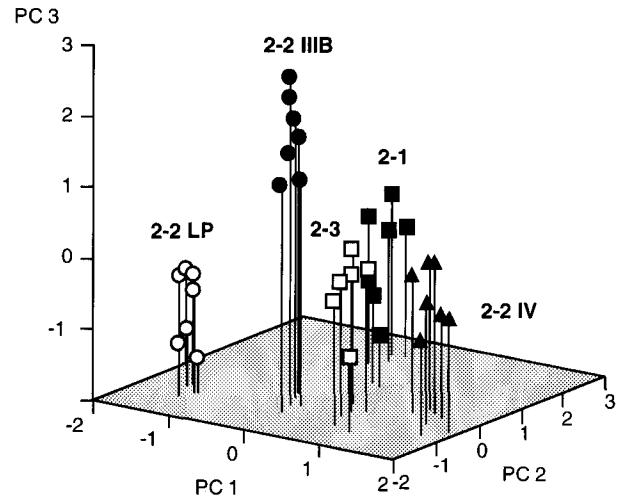

Fig. 1. Plot of the first three principal components (PC 1, PC 2, and PC 3) derived from the fatty acid percent composition of *R. solani* AG 2 isolates, representing AG 2-1 (■), AG 2-2 IIIB (●), AG 2-2 IV (▲), AG 2-2 LP (○), and AG 2-3 (□).

Table 2. Thiamine requirement of *Rhizoctonia solani* AG 2 ISGs.

R. solani ISGs[a]	Dry mycelial weight on the basal medium		P[b]
	with thiamine HCl	without thiamine HCl	
AG 2-1	157.4[c]	148.8	=0.128
AG 2-2 IIIB (mat rush)	174.9	4.9	<0.001
AG 2-2 IV (sugar beet)	193.2	4.6	<0.001
AG 2-2 LP (turf grass)	81.9	5.1	<0.001
AG 2-3 (soybean)	186.1	4.9	<0.001

a) Seven isolates each of AG 2-1, AG 2-2 IIIB, AG 2-2 IV, and AG 2-2 LP and six isolates of AG 2-3 were used.

b) The probability (P) of obtaining t-test (t=0.05) between basal medium with and without thiamine

c) Mean of three replications for the tested ISGs.

Table 3. Percentage composition of total cellular fatty acids identified for *Rhizoctonia solani* AG 2 ISGs.

R. solani ISGs[a]	Percentage composition of fatty acid																	
	Myristic		Pentadecanoic		Palmitic		Palmitoleic		Heptadecanoic		9-Heptadecenoic		Stearic		Oleic		Linoleic	
AG 2-1	0.40[b]	a[c]	1.46	a	7.16	a	0.83	a	0.34	a	1.15	a	1.59	a	10.81	a	76.25	a
AG 2-2 IIIB	0.93	b	0.45	b	9.25	b	0.38	b	0.72	b	0.74	b	1.29	ab	10.32	b	75.89	a
AG 2-2 IV	0.50	a	0.66	c	7.86	c	0.73	a	0.38	a	0.70	b	1.53	ab	7.54	c	80.10	b
AG 2-2 LP	0.52	a	0.40	b	9.18	b	0.17	b	0.07	c	0.69	b	1.23	b	14.93	d	72.84	c
AG 2-3	0.68	ab	0.53	bc	8.47	d	0.29	b	0.39	a	0.61	b	1.40	ab	9.60	e	78.05	d

a) Seven isolates each of AG 2-1, AG 2-2 IIIB, AG 2-2 IV and AG 2-2 LP and six isolates of AG 2-3 were used.

b) Fatty acids in each ISG are expressed as a percentage of total cellular fatty acid composition. Fatty acids were identified by their retention times on the chromatogram.

c) Values in each column described by different letters mean significant difference for Waller-Duncan K-ratio t-test, $u.c.$ =0.05.
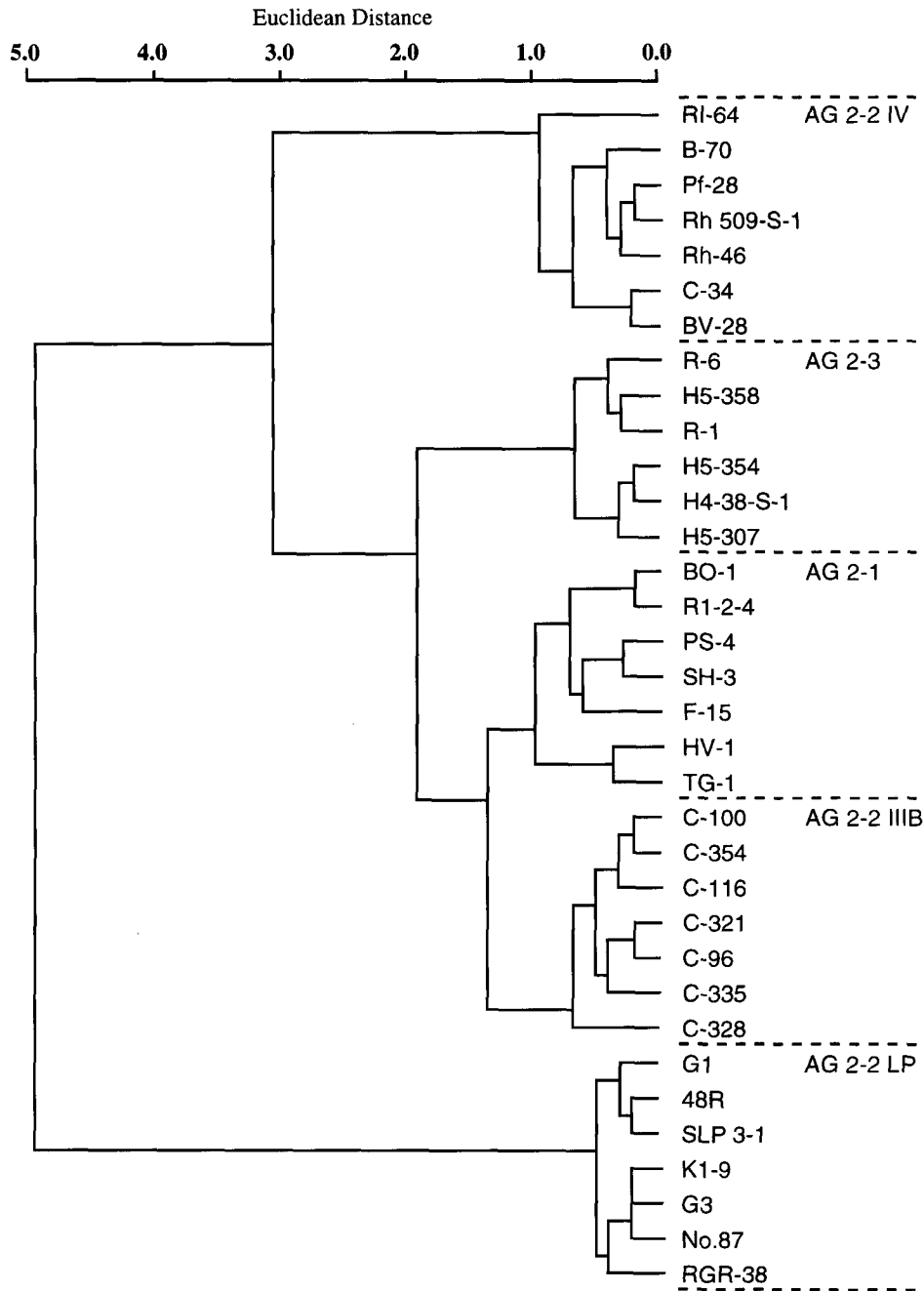
Fig. 2. Dendrogram of cluster analysis on *R. solani* AG 2 isolates, AG 2-1, AG 2-2 IIIB, AG 2-2 IV, AG 2-2 LP, and AG 2-3, based on percent composition of fatty acid.

Discussion

Previous reports that AG 2-2 isolates are auxotrophic for thiamine were confirmed in our study. Isolates of AG 2-2 IIIB, AG 2-2 IV, AG 2-2 LP, and AG 2-3 exhibited 16-42 times more growth in the presence of supplemental thiamine than in its absence.

Our results suggest that analysis of total cellular fatty acids for *R. solani* AG 2-2 ISGs, i.e., AG 2-2 IIIB, AG 2-2 IV, AG 2-2 LP, and AG 2-3, can be used to differentiate pathological types within the anastomosis group. Iso-

lates of AG 2-1 showed similar fatty acid profiles to each other but were distinct from isolates of AG 2-2 ISGs.

Liu and Sinclair (1992) reported the existence of three distinct ISGs and a newly defined group from diseased turfgrass within AG 2-2. In the present study, fatty acid profiles of AG 2-2 LP isolates, which were collected from diseased turfgrass in Japan, were distinct from other AG 2 ISGs in fatty acid profiles, and this result supported the finding of Stevens Johnk and Jones (1993). The difference in fatty acid compositions indicates that the AG 2-2 LP isolates should be regarded as a distinct

4

ISG of AG 2-2.

In Japan, Naito and Kanematsu (1994) reported that a new ISG of AG 2, AG 2-3, caused foliar blight disease of soybean. In the United States, however, isolates from diseased soybean leaves have been placed in AG 2-2 IIIB. The fatty acid profiles of AG 2-3 were closely related to those of AG 2-2 IIIB and AG 2-1 but distinct from those of AG 2-2 LP and AG 2-2 IV in the principal component analysis. Our data support the finding of Stevens Johnk and Jones (1993), who reported a close relationship between AG 2-3 and AG 2-2 IIIB isolates.

Our study of total cellular fatty acid components indicates that the ISGs within AG 2, i.e., AG 2-1, AG 2-2 IIIB, AG 2-2 LP, AG 2-2 IV, and AG 2-3, are genetically separate, representing distinct subgroups within AG 2 isolates of *R. solani*. Comparisons between ISGs in DNA should further reveal their genetic relationships.

Literature cited

Adams, G. C., Jr. 1988. *Thanatephorus cucumeris* (*Rhizoctonia solani*), a species complex of wide host range. In: Advances in plant pathology, vol. 6, Genetics of plant pathogenic fungi, (ed. by Sidhu, G. S.), pp. 535–552. Academic Press, New York.

Burpee, L. and Martin, B. 1992. Biology of *Rhizoctonia* species associated with turfgrass. Plant Dis. **76**: 112–117.

Carling, D. E., Leiner, R. H. and Kebler, K. M. 1987. Characterization of a new anastomosis group (AG-9) of *Rhizoctonia solani*. Phytopathology **77**: 1609–1612.

Haygood, R. A. and Martin, S. B. 1990. Characterization and pathogenicity of species of *Rhizoctonia* associated with centipedegrass and St. Augustinegrass in South Carolina. Plant Dis. **74**: 510–514.

Hurd, B. and Grisham, M. P. 1983. *Rhizoctonia* spp. associated with brown patch of Saint Augustinegrass. Phytopathology **73**: 1661–1665.

Hyakumachi, M., Mushika, T., Ogiso, Y., Toda, T., Kageyama, K. and Tsuge, T. 1998. Characterization of a new culture type (LP) of *Rhizoctonia solani* AG 2-2 isolated from warm-season turfgrasses, and its genetic differentiation from other cultural types. Plant Pathology **47**: 1–9.

Kuninaga, S. and Yokosawa, R. 1980. A comparison of DNA base compositions among anastomosis groups in *Rhizoctonia solani*. Ann. Phytopathol. Soc. Japan **46**: 150–158.

Kuninaga, S. and Yokosawa, R. 1982. DNA base sequence homology in *Rhizoctonia solani* Kühn II. Genetic relatedness within anastomosis group 2. Ann. Phytopathol. Soc. Japan **48**: 668–673.

Liu, Z., Nickrent, D. L. and Sinclair, J. B. 1990. Genetic relationships among isolates of *Rhizoctonia solani* anastomosis group-2 based on isozyme analysis. Can. J. Plant Pathol. **12**: 376–382.

Liu, Z. and Sinclair, J. B. 1991. Isolates of *Rhizoctonia solani* anastomosis group 2-2 pathogenic to soybean. Plant Dis. **75**: 682–687.

Liu, Z. and Sinclair, J. B. 1992. Genetic diversity of *Rhizoctonia solani* anastomosis group 2. Phytopathology **82**: 778–787.

Matsumoto, M., Furuya, N. and Matsuyama, N. 1997. Characterization of *Rhizoctonia* spp., causal agents of sheath diseases of rice plant, by total cellular fatty acids analysis. Ann. Phytopathol. Soc. Japan. **63**: 149–154.

Matsumoto, M., Furuya, N., Takanami, Y., Matsuyama, N., Hyakumachi, M. and Naitou, S. 1996. Characterization of subgroups in AG-2 of *Rhizoctonia solani*. Ann. Phytopathol. Soc. Japan **62**: 294. (In Japanese.)

Matsumoto, T. 1921. Studies in the physiology of the fungi. XII. Physiological specialization in *Rhizoctonia solani* Kühn. Ann. MO Bot. Gard. **8**: 1–62.

Matsuyama, N., Moromizato, Z. Ogoshi, A. and Wakimoto, S. 1978. Grouping *Rhizoctonia solani* Kühn with non-specific esterase zymogram. Ann. Phytopathol. Soc. Japan **44**: 652–658.

Naito, S. and Kanematsu, S. 1994. Characterization and pathogenicity of a new anastomosis subgroup AG 2-3 of *Rhizoctonia solani* Kühn isolated from leaves of soybean. Ann. Phytopathol. Soc. Japan **60**: 681–690.

Ogoshi, A. 1976. Studies on the grouping of *Rhizoctonia solani* Kühn with hyphal anastomosis and on the perfect stages of these groups. Bull. Natl. Inst. Agric. Sci. Ser. C (Plant Pathol. Entomol.) **30**: 1–63.

Ogoshi, A. 1987. Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Ann. Rev. Phytopathol. **25**: 125–143.

Ogoshi, A., Cook, R. J. and Bassett, E. N. 1990. *Rhizoctonia* species and anastomosis groups causing root rot of wheat and barley in the Pacific Northwest. Phytopathology **80**: 784–788.

Ogoshi, A. and Ui, T. 1979. Specificity in vitamin requirement among anastomosis groups of *Rhizoctonia solani* Kühn. Ann. Phytopathol. Soc. Japan **45**: 45–53.

Sherwood, R. T. 1969. Morphology and physiology in four anastomosis groups of *Thanatephorus cucumeris*. Phytopathology **59**: 1924–1929.

Stevens Johnk, J. and Jones, R. K. 1993. Differentiation of population of AG 2-2 of *Rhizoctonia solani* by analysis of cellular fatty acids. Phytopathology **83**: 278–283.

Vilgalys, R. and Gonzalez, D. 1990. Ribosomal DNA restriction fragment length polymorphisms in *Rhizoctonia solani*. Phytopathology **80**: 151–158.

Watanabe, B. and Matsuda, A. 1966. Studies on the grouping of *Rhizoctonia solani* Kühn pathogenic to upland crops. Bull. Appl. Exp. **7**: 1–131.