European Journal of Plant Pathology **103:** 607–622, 1997.

Identification of *Rhizoctonia solani* associated with field-grown tulips using ITS rDNA polymorphism and pectic zymograms

J.H.M. Schneider[1], O. Salazar[2], V. Rubio[2] and J. Keijer[3]
[1]*DLO-Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, NL-6700 GW Wageningen, The Netherlands;* [2]*Centro Nacional de Biotecnología (CSIC-UAM), Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049-Madrid, Spain;* [3]*DLO-State Institute for Quality Control of Agricultural Products (RIKILT-DLO); Address for correspondence: Institute for Sugar Beet Research (I.R.S.), P.O.Box 32, 4600 AA Bergen op Zoom, The Netherlands*

Accepted 7 May 1997

Key words: anastomosis group (AG), sequence alignment

Abstract

Methods based on internal transcribed spacers (ITS) ribosomal DNA (rDNA) polymorphism and pectic zymograms (ZG) were compared for their use in routine identification of *Rhizoctonia solani* isolates occurring in flower bulb fields. Thirty three AG 2-t isolates, pathogenic to tulips, could be distinguished from AG 1-IC, AG 2-2IIIB and AG 2-2IV, AG 3 and AG 5 by means of ITS rDNA fragment length and after digestion with *Eco*R I from AG 4 and AG 5. AG 2-t isolates and two Japanese isolates, pathogenic to crucifers and tulips, had an estimated fragment size of 710 bp, whereas Dutch AG 2-1 isolates, non-pathogenic to tulips, showed an estimated fragment size of 705 bp on agarose gel. Digestion of AG 2-t and AG 2-1 isolates with *Eco*R I, *Sau*3A I, *Hae* III and *Hinc* II revealed four and five distinct ITS rDNA digestion patterns, respectively. In AG 2 isolates 2tR114, 21R14 and 21R61 a double digestion pattern, indicating different ITS sequences within an isolate, was found. The observed ITS fragment length polymorphism between isolates pathogenic and non-pathogenic to tulips were considered too small to be used in routine screening of field isolates. Sequencing of AG 2 isolates 21R01, 21R06, 2tR002 and 2tR144 showed a total ITS rDNA fragment length of 715, 713, 714, and 728 bp. As an alternative to ITS rDNA fragment length polymorphism, pectic enzyme patterns were studied using a commercially available vertical gel-electrophoresis system and non-denaturing polyacrylamide gels amended with pectin. Anastomosis tester isolates AG 1 to AG 11 revealed different ZG. Fifty AG 2-t isolates and five AG 2-1 isolates belonged to a homogeneous pectic zymogram group. We propose to assign AG 2 isolates pathogenic to crucifers and tulip to ZG5-1. AG 2-1 isolates, non-pathogenic to tulip, formed a heterogeneous group with 4 distinct ZG. Pectic zymography provides an easy, quick and unambiguous method for routine identification of large numbers of field isolates. Such a technique is needed for research on the dynamics of *Rhizoctonia* populations to develop environmentally friendly control measures of rhizoctonia disease in field-grown flower bulbs.

Introduction

Rhizoctonia solani Kühn (*Thanatephorus cucumeris* (Frank) Donk) is a destructive soilborne plant pathogen affecting many agricultural crops worldwide (Parmeter, 1970; Ogoshi, 1987). In the Netherlands, the fungus is an important pathogen of potato and sugarbeet, and, during the last three decades, the pathogen has become an increasing problem in field-grown tulips. For environmental reasons, current chemical control has to be reduced and alternative control measures based on the ecology of the pathogen are being developed.

At present, isolates of *R. solani* are identified according to hyphal fusion with anastomosis tester isolates. Isolates are assigned to one of the current 12 anastomosis groups (AG), designated AG 1 to AG 11 and AG BI (Sneh et al., 1991; Carling et al., 1994).

AG are to some extent associated with cultural characteristics and host range, but within AG considerable variability occurs. AG can be subdivided according to host range, thiamine requirement and DNA homology. In total, 21 AG and subgroups have been identified (Sneh et al., 1996). AG 2 is the only AG of *R. solani* subdivided according to hyphal fusion frequency (FF) (Ogoshi, 1975; Carling and Sumner, 1992). A FF of 50% and more is regarded as high FF and it occurs only between isolates belonging to the same AG subgroup, whereas a FF of less than 30%, regarded as low FF, may occur between isolates representing different AG subgroups. To date, AG 2-1, AG 2-2 (Ogoshi, 1987), and AG 2-3 (Naito and Kanematsu, 1994) are recognized. Hyphae of isolates of AG 2-2 fuse in low frequency with isolates representing AG 2-1 and AG 2-3. Isolates representing AG 2-3 fuse in high frequency with AG 2-1 isolates, but are designated to AG 2-3 because of thiamine requirement and the host range.

Rhizoctonia disease of flower bulbs may be caused by several anastomosis groups. Isolates of AG 2-t, AG 2-2, AG 4 and AG 5 have been reported pathogenic to flower bulbs (Doornik, 1981; Schneider et al., 1997). Typical bare patch symptoms in field-grown tulips are caused by a subgroup of *R. solani*, designated as AG 2-t (Schneider et al., 1997). These AG 2-t isolates affect both cruciferous crops and flower bulbs and, according to FF, are closely related to Dutch AG 2-1 isolates which affect cruciferous crops but not flower bulbs. Single *R. solani* AG 2-t isolates cannot be distinguished conclusively from AG 2-1 isolates due to high variability in FF. Since pathogenicity tests are laborious and time consuming, alternative identification methods for AG 2-t isolates are required.

Molecular approaches for identification of isolates of *R. solani* include genomic restriction fragment length polymorphism (Vilgalys and Gonzalez, 1990; Jabaji-Hare et al., 1990; O'Brien, 1994), random amplified polymorphic DNA (Duncan et al., 1993; Yang et al., 1995), polymorphism of the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) (Liu et al., 1992, 1993, 1995; Liu and Sinclair, 1993; Kanematsu and Naito, 1995), and pulsed field gel electrophoresis (Keijer et al., 1996). Liu et al. (1992) studied ITS rDNA polymorphism of AG 2 and distinguished AG 2-1, AG 2-2IIIB and AG 2-2IV, and two additional subgroups, 2D and 2E. Later, 2E was assigned to AG 3 (Stevens Johnk et al., 1993). Kanematsu and Naito (1995) used ITS rDNA polymorphism to demonstrate AG 2-3 being a genetically distinct subgroup within AG 2. The techniques mentioned revealed a high level of variation between AG, with potential for development of specific markers for AG.

Another technique, pectic zymograms (ZG), has been applied successfully to distinguish subgroups within AG 8. A pectic zymogram comprises a pattern of polygalacturonases, pectine esterases and lyases (Cruickshank and Wade, 1980). The ZG coding system is not analoguous to the AG coding system. For an overview of ZG within AG see MacNish et al. (1994). Within AG 8, ZG 1-1 to 1-5 are distinguished which seem to be related to host range and specific disease symptoms in the field (Sweetingham et al., 1986; Neate et al. 1988; MacNish and Sweetingham, 1993). ZG1-1 to ZG1-5 cannot be distinguished by hyphal fusion frequency (Neate et al., 1988). Pectic zymograms are stable and reproducible (MacNish and Sweetingham, 1993), and relatively easy to accomplish. Cruickshank (1990) reported zymogram patterns for AG 1 to AG 7 and AG BI, including one isolate designated as AG 2-3. This isolate was obtained from cold glasshouse-grown tulips in the Netherlands and should not be confused with the AG 2-3 isolates reported by Naito and Kanematsu (1994). The zymogram pattern of the tulip AG 2-3 isolate differed from the pattern of the tested AG 2-1 isolates. Further use of pectic zymograms for characterization of field isolates of *R. solani* has thus far been limited to Australia because of the specifically designed equipment which is not commercially available.

A detailed study of the ecology and the population dynamics of bare patch caused by AG 2-t isolates in tulip needs an unambiguous identification method. Pathogenicity tests are time-consuming and too costly to screen the numerous isolates obtained from field-grown flower bulbs. The present paper describes the possible use of ITS rDNA polymorphism and an adapted pectic zymography method for the identification of AG 2-t isolates in routine screening procedures.

Materials and methods

Fungal isolates

A summary of characteristics of *R. solani* isolates used in this study is presented in Tables 1 and 2. Pure cultures were maintained on Malt Peptone Agar (MPA) slants at 10 °C. MPA contained 15 g Malt extract (Oxoid L39), 1.25 g special peptone (Oxoid L72), and 15 g technical agar (Oxoid L13) per litre of tap water (Van den Boogert and Jager, 1984). Prior to

Table 1. Codes and origins of *Rhizoctonia solani* AG 2-t isolates used in this study

IPO-code[1]	Cultivar[2]	Site of isolation	Location in the Netherlands
2tR002	Red Riding Hood	Stem	Noordwijkerhout
2tR003	Balalaika	Stem	Noordwijkerhout
2tR004	*Tulipa kaufmanniana*	Stem	Hillegom
2tR005	Tulip	Stem	Hillegom
2tR006	Verdi	Leaf	Hillegom
2tR007[3]	Golden Apeldoorn	Bulb	Honselaarsdijk
2tR008	Tulip	Stem	unknown
2tR009	Apeldoorn	Stem	Heemskerk
2tR101	Varinas	Leaf	Breezand
2tR102	Varinas	Stem	Breezand
2tR103	Halcro	Leaf	Breezand
2tR104	Halcro	Stem	Breezand
2tR105	Menton	Stem	Breezand
2tR106	Menton	Soil	Breezand
2tR107	Estella Rijnveld	Stem	Julianadorp
2tR108	Giant Parrot	Bulb	Noordwijkerhout
2tR109	Giant Parrot	Stem	Noordwijkerhout
2tR110	Giant Parrot	Soil	Noordwijkerhout
2tR111	Inzell	Leaf	Noordwijkerhout
2tR112	Inzell	Soil	Noordwijkerhout
2tR113	*Iris* 'White Excelsior'	Stem	Heemskerk
2tR114	*Iris* 'White Excelsior'	Bulb	Heemskerk
2tR115	*Iris* 'White Excelsior'	Soil	Heemskerk
2tR116	*Ixia* spp.	Bulb	Heemskerk
2tR117	*Ixia* spp.	Stem	Heemskerk
2tR118	Leen van der Mark	Leaf	Castricum
2tR119	Leen van der Mark	Stem	Castricum
2tR120	Leen van der Mark	Bulb	Castricum
2tR121	Leen van der Mark	Soil	Castricum
2tR122	Judith Leyster	Leaf	St. Pancras
2tR123	Judith Leyster	Stem	St. Pancras
2tR124	Judith Leyster	Bulb	St. Pancras
2tR125	Gander	Leaf	Breezand
2tR126	Gander	Stem	Breezand
2tR127	Gander	Soil	Breezand
2tR128	Pink Supreme	Leaf	Anna Paulowna
2tR129	Plaisir	Leaf	Anna Paulowna
2tR130	Polo	Leaf	Lisse
2tR133	Hurts Delight	Stem	De Zilk
2tR134	Hurts Delight	Bulb	De Zilk
2tR135	Red Riding Hood	Leaf	De Zilk
2tR136	Plaisir	Stem	De Zilk
2tR137	Ali Baba	Bulb	De Zilk
2tR138	Red Riding Hood	Leaf	Den Helder
2tR139	Red Riding Hood	Bulb	Den Helder
2tR140	Pinoccio	Bulb	Anna Paulowna
2tR142	Red Riding Hood	Leaf	Lisse
2tR143	Red Riding Hood	Bulb	Lisse
2tR144	Fashion	Stem	Lisse
2tR145	*Hyacinthus* spp.	Bulb	Lisse
2tR146	*Hyacinthus* spp.	Stem	Lisse

[1]All isolates were collected by J.H.M. Schneider, except isolates 2tR002 through 2tR009 which correspond to LBO codes T8, T10, T11, T12, T13 and PD codes PD 83/114 (CBS 343.84), PD 88/315, PD 82/37, respectively. LBO: Bulb Research Centre, Lisse, the Netherlands; PD: Plant Protection Service, Wageningen, the Netherlands; CBS: Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.
[2]Tulip cultivars unless otherwise mentioned.
[3]All isolates displayed the same ZG5-1 pattern, except for isolate 2tR007.

further procedures, isolates were transferred via tap water agar amended with 250 ppm chloramphenicol to a defined nutrient medium (DNM) (Keijer et al., 1996). DNM-medium contained 15 g sucrose, 2 g asparagine, 0.6 g KH_2PO_4, 0.8 g K_2HPO_4, micro salts (2.5 μg $ZnSO_4$, 2.5 μg H_3BO_3, 0.5 μg $MnSO_4.1H_2O$, 1.7 μg NaFeEDTA, 0.3 μg $CuSO_4.5H_2O$ and 0.1 μg $(NH_4)_6Mo_7O_{24}.4H_2O$), 0.01 g $CaCl_2$, 1 g $MgSO_4$, and vitamins (1 μg thiamin, 1 μg niacin, 20 ng biotin, 0.05 μg Ca-pantothenate, 0.5 μg pyridoxine, and 0.1 μg ρ-aminobenzoic acid) per litre of ddH_2O. A solid medium, DNMA, was prepared by adding 1.5% (w/v) technical agar.

Isolation of DNA

Thirty three arbitrarly chosen *R. solani* isolates (Table 1) were transferred from DNMA-plates to a 24 well tissue-culture plate with 1 ml DNM per well and incubated at 23 °C for 3–7 days. For each isolate, DNA was isolated as described in Keijer et al. (1996). The mycelium was freeze-dried overnight, pulverized with a pestle in a reaction tube, suspended in extraction buffer (50 mM Tris-HCl, 100 mM EDTA and 1% sodium dodecyl sulphate), and incubated on ice for 5 min. Proteins and cell debris were precipitated by centrifugation for 10 min at 14000 rpm (Eppendorf 5414S). The supernatant was transferred to a new tube and extracted once with phenol-chloroform-isoamylalcohol (25:24:1) and once with chloroform-isoamylalcohol (24:1) (Sambrook et al., 1989). RNA was removed by treating the samples with RNAse A (final concentration 50 μg/ml) for 30 min at 37 °C. DNA was precipitated by adjusting the samples to

Table 2. Codes, anastomosis group (AG), pectic zymogram group (ZG) and origin of *Rhizoctonia solani* isolates AG 1 to AG 11 used in this study

IPO-code	AG and subgroup	ZG	Host	Origin	Original isolate designation	Source[1]
01R01	1-IA	11-1	Rice	Japan	CS-KA	Ogoshi
01R02	1-IB	11-2	Sugar beet	Japan	B-19	Ogoshi
01R03	1-IC	11-3	Sugar beet	Japan	BV-7	Ogoshi
01R11	1	11-3	Gladiolus	the Netherlands	PD 77/679	PD
01R21	1	11-2	Carrot	the Netherlands	PD 83/698	PD
01R31	1	11-2	Lettuce	the Netherlands	PD 80/426	PD
01R41	1	11-2	Grass	the Netherlands	PD 82/674	PD
21R01	2-1	5-1	Pea	Japan	PS-4	Ogoshi
21R04	2-1	5-1	Barley	Japan	HV	PD
21R06	2-1	5-1	Tulip	Japan	TG-1	Ogoshi
21R11	2-1	5-2	Cauliflower	the Netherlands	PD 80/710	PD
21R12	2-1	5-2	Cauliflower	the Netherlands	PD 81/228	PD
21R14	2-1	5-2	Cauliflower	the Netherlands	PD 86/723	PD
21R21	2-1	5-2	Cauliflower	the Netherlands	PD 86/748	PD
21R31	2-1	5-3	Swede	the Netherlands	PD 84/36	PD
21R41	2-1	5-2	Swede	the Netherlands	PD 81/130	PD
21R51	2-1	n.a.[2]	Oil-seed rape	the Netherlands	PD 80/664	PD
21R61	2-1	5-3	Lily	the Netherlands	PD 83/866	PD
21R71	2-1	5-3	Turnip rape	the Netherlands	PD 83/010	PD
21R81	2-1	5-4	Lettuce	the Netherlands	PD 83/909	PD
21R91	2-1	n.a.	Leek	the Netherlands	PD 83/303	PD
21R92	2-1	n.a.	unknown	the Netherlands	PD 84/376	PD
21R93	2-1	5-1	Potato	Alaska	F56L	Carling
22R01	2-IIIB	10-1	Mat rush	Japan	C-96	ATCC 76124
22R02	2-2IV	10-2	Sugar beet	Japan	RI-64	ATCC 76125
22R10	2-2	n.a.	Sugar beet	the Netherlands	PD 83/585	PD
22R11	2-2	n.a.	Sugar beet	the Netherlands	PD 85/904	PD
22R13	2-2	10-1	Vriesia	the Netherlands	PD 83/327	PD
22R15	2-2	10-2	Cactus	the Netherlands	PD 83/774	PD
22R16	2-2	10-2	Calluna	the Netherlands	PD 83/842	PD
22R17	2-2	n.a.	Maize	the Netherlands	PD 89/956	PD
23R01	2-3	6-1	Soybean	Japan	R-6	Naito
23R02	2-3	6-1	Soybean	Japan	H4-38-S-1	Naito
23R03	2-3	6-1	Soybean	Japan	H5-307	Naito
03R01	3	7	Potato	Japan	ST-11-6	Ogoshi
03R03	3	7	Potato	the Netherlands	36 AB65	Jager
03R04	3	7	Potato	the Netherlands	09 ABa	Jager
03R05	3	7	Potato	the Netherlands	05 AHa	Jager
03R06	3	7	Potato	Japan	ST9	PD
03R08	3	7	Potato	Germany	CBS 363.82	Keijer
03R09	3	7	Potato	Norway	R3/Sundheim	Keijer
03R12	3	7	Potato	the Netherlands	PD 80/102	PD
03R13	3	7	Potato	the Netherlands	PB3	Van den Boogert
03R14	3	7	Potato	the Netherlands	3R11	Keijer
03R15	3	7	Potato	the Netherlands	3R12	Keijer
03R16	3	7	Potato	the Netherlands	3R13	Keijer

Table 2. Continued

IPO-code	AG and subgroup	ZG	Host	Origin	Original isolate designation	Source[1]
04R02	4 HG-I	8-1	Peanut	Japan	AH-1	Ogoshi
04R03	4 HG-II	8-2	Sugar beet	Japan	Rh-165	Ogoshi
04R06	4	n.d.[3]	Iris	the Netherlands	I2	LBO
04R08	4	n.a.	Iris	Israel	I Yot	LBO
04R09	4	n.a.	Lettuce	the Netherlands	S1	LBO
04R10	4	n.a.	Lettuce	the Netherlands	S2	LBO
04R11	4	8-2	Bean	the Netherlands	PD 82/576	PD
04R22	4	8-2	Iris	the Netherlands	I7	LBO[2]
04R61	4	n.a.	Spinach	the Netherlands	PD 83/418	PD
04R70	4	n.d.	Anemone	the Netherlands	PD 84/659	PD
04R71	4	n.a.	Cucumber	the Netherlands	PD 84/762	PD
04R90	4	8-1	Spinach	USA	APK-SP1	Keinath
04R91	4	8-1	Tomato	Bulgary	149	PD
04R92	4	8-1	Tomato	Bulgary	106C3	PD
05R01	5	12	Soybean	Japan	GM-10	Ogoshi
05R02	5	12	Soil	Japan	CBS 143.82	PD
05R06	5	12	Lily	the Netherlands	PD 82/741	PD
05R07	5	12	Iris	the Netherlands	PD 82/384	PD
05R21	5	12	Maize	the Netherlands	PD 83/481	PD
05R31	5	12	Soil	the Netherlands	PD 84/865	PD
06R01	6 HG-I	13	Soil	Japan	OMT-1-1	Ogoshi
06R02	6 GV	13	Soil	Japan	NKN-2-1	Ogoshi
07R01	7	14	Soil	Japan	HO-1556	Ogoshi
08R15	8	1-1	Wheat	Western Australia	93087	MacNish
08R23	8	1-2	Barley	Western Australia	92630	MacNish
08R31	8	1-3	Barley	South Australia	1512	MacNish
08R45	8	1-4	Barley	Western Australia	93305	MacNish
08R51	8	1-5	Triticali	Western Australia	91069	MacNish
09R10	9	15-1	unknown	Alaska	V12M	Carling
09R12	9	15-2	unknown	Alaska	F57M	Carling
10R01	10	9	Barley	USA	w-395	ATCC 76107
11R02	11	3	Lupine	Western Australia	R1352	Carling

[1]PD: Plant Protection Service, Wageningen, the Netherlands; LBO: Bulb Research Centre, Lisse, the Netherlands.
[2]n.a.: Not assigned to ZG.
[3]n.d.: Not determined.

250 mM NH_4-acetate, addition of 2 vol of ice cold 96% ethanol, incubation for 10 min at –80 °C and centrifugation for 20 min at 4 °C. The pellet was rinsed with ice cold 70% ethanol and centrifuged for 20 min at 4 °C. After drying, the DNA was resuspended in 50 μl ddH_2O and stored until further use at –20 °C.

ITS rDNA polymorphism

DNA amplification was slightly modified after Innis and Gelfand (1991) and Liu et al. (1992). PCR reaction tubes contained 1.5 mM $MgCl_2$, buffer (50 mM KCl, 10 mM Tris/HCl (pH 8.3)), 0.05% W1 (Life Science Technologies, Bethesda, MD), 60 μM of each dATP, dCTP, dGTP, dTTP, 0.6 mM each of primer ITS1 5′(TCCGTAGGTGAACCTGCGG)3′ and ITS4 5′(TCCTCCGCTTATTGATATGC)3′ (White et al., 1991; Liu et al., 1992), 2 U Taq-DNA polymerase (Life Science Technologies (510-8038 SD)), and approximately 1–10 ng *R. solani* DNA. In all PCR-reaction sets a negative control without DNA was included.

PCR reaction mixtures were covered with oil to prevent evaporation and incubated in a thermal cycler (Perkin Elmer Cetus) using the following conditions:

DNA-denaturization for 2 min at 94 °C, 30 cycles of DNA-denaturization for 1 min at 94 °C, primer annealing for 1 min at 57 °C and primer extension for 2 min at 72 °C. DNA-amplification was terminated by primer extension for 10 min at 72 °C and final incubation at 4 °C. DNA-amplification products (10 μl per sample) were loaded on a standard agarose gel (1.5%), separated by electrophoresis in 0.5x TBE buffer (45mM Tris-borate and 1 mM EDTA), stained with ethidium bromide, and visualised under UV light (Sambrook et al., 1989).

Differentiating digestion patterns of ITS rDNA fragments between anastomosis groups and between anastomosis subgroups were screened for by using restriction endonucleases *Alu* I, *Bam*H I, *Bgl* II, *Cla* I, *Dde* I, *Dra* I, *Eco*R I, *Eco*R V, *Hae* III, *Hinc* II, *Hinf* I, *Hha* I, *Kpn* I, *Mbo* I, *Msp* I, *Pst* I, *Pvu* II, *Rsa* I, *Sau*3A I, *Sst* I, *Sst* II, *Sty* I, *Taq* I, *Xba* I, and *Xho* I under conditions as recommended by the manufacturer (Life Science Technologies or New England Biolabs). DNA restriction fragments were separated using agarose gel electrophoresis (Sambrook et al., 1989) and visualised as described above.

Sequence analysis of the ITS region

R. solani isolates 21R01, 21R06, 2tR002 and 2tR144 were used to study genetic variation in the ITS region as described previously for AG 4 (Boysen et al., 1996). First, single stranded DNA was obtained by asymmetric PCR and the use of primers ITS4 and ITS5 5′(GGAAGTAAAAGTCGTAACAAGG)3′ in a 50:1 ratio (Gyllensten and Ehrlich, 1988), and alternatively, ITS1F 5′(CTTGGTCATTTAGAGGAAGTAA)3′ and ITS4B 5′(CAGGAGACTTGTACACGGTC)3′ (Gardes and Bruns, 1993). For each reaction, a 50 μl mixture contained 10 ng genomic DNA, 20 pmol one primer, 0.4 pmol another primer, 1.25 U Taq DNA polymerase (USB, Cleveland Ohio), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP (Pharmacia, Sweden), 0.0025% Tween 20, and 10% dimethylsulphoxide (DMSO) in ddH_2O and was covered with mineral oil. Amplification was performed in an automated thermal cycler (Perkin Elmer Cetus Corp.) using the following conditions: initial denaturation for 2.5 min at 94 °C, followed by 40 cycles of denaturation for 15 sec at 94 °C, annealing for 30 sec at 53 °C for ITS4/ITS5 and at 55 °C for ITS1F/ITS4B, and 1.5 min extension at 72 °C. DNA-amplification was terminated by a final extension for 10 min at 72 °C and incubation at 4 °C. Prior to sequencing, excess primers and nucleotides were removed from the asymmetric amplification mixture by precipitation for 10 min at room temperature with an equal volume of 5M NH_4-acetate and 2.5 vol of 96% ethanol and pelleting by centrifugation. The PCR products were washed with ice cold 80% ethanol, dried at 37 °C and finally resuspended in 17 ml TE buffer, after which the DNA was used for sequencing without further purification. Sequencing was done by the Sanger's dideoxy method (Sanger et al., 1977) using the Sequenase Version 2.0 sequencing kit (USB, Cleveland Ohio), single stranded PCR (ss-PCR) products as templates and ITS1, ITS2 5′(GCTGCGTTCTTCATCGATGC)3′, ITS3 5′(GCATCGATGAAGAACGCAGC)3′ and ITS4 as sequencing primers. Approximately 1 μg ss-PCR product and 50 ng primer were used for each reaction and labelling was done with (α-^{35}S)-dATP (Amersham Int., Amersham). The sequence fragments were separated in a denaturating 8.3 M urea/6% polyacrylamide gel.

Pectic zymograms

Pectic enzymes were induced by growing 51 *R. solani* isolates (Table 1) in 6 well plates, each well containing 5 ml of culture medium slightly modified after Sweetingham et al. (1986). The culture medium contained 2.64 g $(NH_4)_2SO_4$, 0.34 g K_2HPO_4, 1.14 g $MgSO_4.7H_2O$, 10 g citrus pectin (Sigma P 9135 with 84% galacturonic acid and a methoxy content of 9.4%), 0.01 g $CaCl_2$ and vitamins as for the DNM medium per litre of ddH_2O. The pH of the medium was adjusted to 5.5 with NaOH. After 5–14 days of incubation at 20 °C in the dark, culture fluids were pipetted into a reaction tube and stored at –20 °C.

Non-denaturating polyacrylamidegel-electrophoresis, modified after Laemmli (1970) and Cruickshank and Wade (1980), was done using a Biorad mini Protean II gelsystem as follows: the separating gel contained 10% (w/v) acrylamide-bisacrylamide (36.5:1) and 0.1% (w/v) citrus pectin in 1.5 M Tris-HCl (pH 8.8). To facilitate polymerization, 3% Ammoniumpersulphate (APS) and 0.035% TEMED (6 M N,N,N',N'-tetramethylethylenediamine) were added. The stacking gel contained 3% (w/v) acrylamide-bisacrylamide (36.5:1), 0.1% citrus pectin in 0.5 M Tris-HCl (pH 6.8), 3% APS and 0.035% TEMED. Samples consisted of 20 μl culture supernatant sample and 5 μl sample buffer (0.1 M Tris/HCl pH 6.8, 18% v/v glycerol, 0.01% w/v bromophenol blue). Electrophoresis was done for 60 min at 200 V with 250 mM

Tris and 1.92 M Glycine (pH 8.3) as electrophoresis buffer. After electrophoresis, gels were incubated for 90 min in 0.1 M DL-malic acid (Sigma M-0875) and stained for 2 h in 0.05% ruthenium red (Sigma R 2751) and destained overnight in 3 mM Na_2CO_3. On each gel *R. solani* isolate 2tR002 was used as a reference. Gels were photographed, dried and sealed in cellophane (Sambrook et al., 1989). For ease of comparison with the ZG obtained with a horizontal gel system and shown in negative contrast (Cruikshank, 1980; Sweetingham et al., 1986), our gels also are displayed in negative contrast.

Results

ITS rDNA polymorphism

After amplification of the ITS rDNA using primers ITS1 and ITS4 the undigested DNA fragment lengths of the tested isolates varied from 685 to 740 bp (Table 3). One isolate representing AG 2-t, with an estimated fragment length of 710 bp, could clearly be distinguished from anastomosis tester isolates representing AG 1-IC (685 bp), AG 2-2IIIB (740) and 2-2IV (740 bp), AG 3 (700 bp), and AG 5 (690 bp). Estimated fragment lengths for anastomosis tester isolates AG 1-IA and IB, AG 2-1, and AG 4 and the AG 2-t isolate were approximately 710 bp under our laboratory conditions. After digestion with *Eco*R I, AG 2-t isolates could readily be distinguished from the AG 1 and AG 4 isolates. The AG 2-t isolate rendered the same digestion pattern as AG 2-1. An overview of digestion patterns and estimated fragment lengths is presented in Table 3. Endonucleases *Bam*H I, *Bgl* II, *Eco*R V, *Kpn* I, *Pst* I, *Rsa* I, *Sst* I, *Sst* II, *Xba* I, and *Xho* I were not able to digest the amplified ITS region.

Comparison of 33 AG 2-t and 14 AG 2-1 isolates showed 2 Japanese isolates, 21R01 and 21R06, and 32 AG 2-t isolates with an estimated rDNA fragment size of 710 bp and 12 Dutch AG 2-1 isolates and 2tR128 with an estimated fragment size of 705 bp. No length polymorphism of amplified rDNA products was observed within the screened Dutch AG 2-1 and AG 2-t isolates, except for 2tR128. Within the AG 2-t and AG 2-1 isolates four and five ITS-types, respectively, could be distinguished after digestion with *Eco*R I, *Sau*3A I, *Hae* III and *Hinc* II (Figure 1; Table 4). Japanese isolates 21R01 and 21R06 revealed the same ITS-type as AG 2-t isolates. Digestion with *Eco*R I and *Sau*3A I yielded ITS rDNA-fragments for all tested AG 2-t and AG 2-1 isolates, with slightly different fragment sizes, thus distinguishing AG 2-t, 21R01, 21R06 and 21R51 from other AG 2-1 isolates and 2tR128. Isolate 2tR144 showed a double digestion pattern when digested with *Hinc* II. Isolates 2tR128 and 21R81 showed the same digestion pattern for *Eco*R I and *Hae* III, thus distinguishing these isolates from AG 2-t and AG 2-1 isolates. Isolate 2tR128 can be distinguished from 21R81 by *Hinc* II. Isolate 2tR007 revealed a pattern different from the other isolates after digestion with *Hae* III.

Within the 12 Dutch AG 2-1 isolates five ITS-types were distinguished according to restriction fragment sizes. Use of *Hinc* II revealed heterogeneous fragment digestion patterns in isolates 21R14, 21R31, 21R61, 21R71, 21R92, whereas in isolates 21R11, 21R12, 21R21, 21R41, 21R51, 21R81, and 21R91 no digestion occurred.

R. solani AG 2-2 isolates 22R01 and 22R02 showed an estimated ITS rDNA fragment length of 740 bp. Endonuclease *Msp* I differentiated AG 2-2IIIB (740 bp) from AG 2-2IV (580 and 160 bp) (Table 3). Isolates 22R10, 22R11, 22R15, and 22R16 also had a rDNA fragment length of 740 bp, whereas isolate 22R17 had a smaller (715 bp) rDNA fragment (data not shown). Isolates 22R10, 22R11, 22R15 and 22R16 showed two restriction fragments (580 and 160 bp) after digestion with *Msp* I. Isolate 22R13 was not digested by *Msp* I (data not shown).

The estimated length of the amplified rDNA fragment of AG 4 was approximately 710 bp for all tested isolates except for isolate 04R11, with an estimated ITS fragment length of 720 bp. Digestion with *Hinc* II and *Hae* III revealed three subgroups within AG 4. *Hinc* II gave two digestion patterns representing AG 4 HG-I and AG 4 HG-II. *Hae* III also showed two digestion patterns of which the *Hae* III pattern for 04R03 also occurred for isolates 04R70 and 04R71 (Table 4).

The estimated length of the amplified rDNA fragment was 700 bp for the AG 3 and 690 bp for the AG 5 isolates. No rDNA polymorphism was observed after digestion with endonucleases *Hae* III, *Sau*3A I and *Hha* I among the AG 3 and AG 5 isolates listed in Table 2. Of the tested endonucleases *Dra* I did not digest any of the tested AG 5 isolates.

Alignment of sequences

Direct sequencing of PCR products showed a total ITS length of 715, 713, 714, and 728 bp for Japanese isolates 21R01 and 21R06 and Dutch AG-2-t isolates

Table 3. Estimated length of digestion fragments of the ITS-region of *Rhizoctonia solani* AG 2-t and anastomosis tester isolates

AG isolate enzyme	1-IA 01R01	1-IB 01R02	1-IC 01R03	2-1/2-t 21R01/ 2tR005	2-2IIIB 22R01	2-2IV 22R02	3 03R01	4 HG-I 04R02	4 HG-II 04R03	5 05R01
Msp I	710	710	685	710	740	580 160	700	710	710	690
Hae III	525 120 65	525 120 65	395 120 105 65	525 120 65	610 130	610 130	580 120	590 120	590 120	570 120
Hinc II	710	710	685	445 265	460 280	460 280	430 270	430 280	710	425 265
*Eco*R I	375 335	365 345	365 320	345 285 80	380 360	380 360	360 340	370 340	370 340	355 335
Cla I	425 285	410 300	415 270	410 300	425 315	425 315	410 290	420 290	420 290	405 285
Hinf I	370 340	355 355	360 325	355 355	375 365	375 365	350 350	365 345	365 345	350 340
*Sau*3A I	270 255 145 40	275 250 145 40	260 240 145 40	275 250 145 40	280 260 160 40	280 260 160 40	265 250 145 40	265 255 150 40	265 255 150 40	255 250 145 40
Dde I	280 270 140 20	275 275 140 20	275 250 140 20	275 275 140 20	295 265 160 20	295 265 160 20	270 270 140 20	270 270 150 20	270 270 150 20	265 265 140 20
Alu I	490 220	330 220 100 70	465 220	420 220 70	430 240 70	430 240 70	420 220 70	410 230 70	410 230 70	400 220 70
Hha I	370 340	375 335	350 335	375 335	390 350	390 350	365 335	365 345	365 345	355 345
Dra I	480 230	475 235	475 210	475 235	500 205 35	500 205 35	475 225	485 225	485 225	690
Sty I	385 205 120	395 195 120	270 205 120 90	390 200 120	400 215 125	400 215 125	380 200 120	380 210 120	380 210 120	370 200 120
Taq I	360 295 55	355 300 55	360 270 55	355 300 55	375 310 55	375 310 55	355 290 55	365 290 55	365 290 55	350 285 55

Table 4. Estimated length of digestion fragments of the ITS-region of *Rhizoctonia solani* AG 2-t, AG 2-1 and AG 4 isolates

AG	ITS-type[1]	restriction enzyme				Isolates
		*Eco*R I	*Hae* III	*Sau*3A I	*Hinc* II	
2-t	I	345	525	275	440	2tR002 2tR101 2tR107 2tR118 2tR127 2tR135 21R01
		285	120	250	270	2tR003 2tR102 2tR112 2tR120 2tR129 2tR139 21R06
		80	65	145		2tR004 2tR104 2tR114 2tR124 2tR130 2tR142
				40		2tR005 2tR105 2tR116 2tR125 2tR133 2tR143
						2tR008 2tR106 2tR117 2tR126 2tR134 2tR145
	II	345	525	275	710	2tR144
		285	120	250	440	
		80	65	145	270	
				40		
	III	370	585	270	435	2tR128
		335	120	250	270	
				145		
				40		
	IV	330	585	270	705	2tR007
		295	520	250		
		80	120	145		
			65	40		
2-1	I	330	520	270	705	21R12
		295	120	250		21R21
		80	65	145		21R41
				40		21R91
	II	330	520	270	705	21R14 21R71
		295	120	250	435	21R31 21R92
		80	65	145	270	21R61
				40		
	III	330	705	270	705	21R11
		295		250		
		80		145		
				40		
	IV	330	520	275	705	21R51
		295	120	250		
		80	65	145		
				35		
	V	370	585	270	705	21R81
		335	120	250		
				145		
				40		
4	HG-I	n.d.[2]	590	n.d.	430	04R02 04R61
			505		280	04R08 04R91
			120			04R09
			85			04R10
	HG-II	n.d.	590	n.d.	710	04R03
			120			04R22

Table 4. Continued

AG	ITS-type[1]	restriction enzyme				Isolates
		*Eco*R I	*Hae* III	*Sau*3A I	*Hinc* II	
	III	n.d.	590	n.d.	430	04R70
			120		280	04R71
	IV	n.d.	605	n.d.	720	04R11
			115			

[1]The authors do not intend to present these ITS types as subgroups of AG, but use the codes in this paper for convenience only.
[2]n.d.: Not determined.

2tR002 and 2tR144, respectively. The ITS-b length was 273 nucleotides for isolates 21R01, 21R06, and 2tR002 and 279 bp for isolates 2tR144 (Figure 3). Furthermore, differences in nucleotides were observed thus enabling to distinguish two AG 2-t isolates from two Japanese AG 2-1 isolates.

Pectic zymograms

The AG 2-t isolates except for 2tR007 (Table 1) and isolates 21R01, 21R04, 21R06, and 21R93 (Table 2) formed a homogeneous zymogram group (Tables 1 and 2; Figure 2). Twelve Dutch AG 2-1 isolates formed a heterogeneous group with five distinct ZG (Table 2; Figure 2). Isolate 2tR007 revealed a ZG different from these AG 2-t and AG 2-1 isolates (Figure 2).

Using the zymogram technique, AG 1 isolate 01R11 could be assigned to AG 1-IC, and isolates 01R21, 01R31 and 01R41 to AG 1-IB (Table 2). Among the tested AG 2-2 isolates, two ZG represented the known AG 2-2IIIB and AG 2-2IV (Table 2). Two other isolates showed a different ZG pattern but remained unassigned to a ZG (Table 2). Within AG 4 two ZG could be distinguished in accordance with rDNA homology subgrouping (Kuninaga and Yokosawa, 1984) and some isolates showed a different ZG pattern which remained unassigned (Table 2). No ZG heterogeneity was observed within the tested AG 3 and AG 5 isolates. Isolates of AG 6 to AG 11 showed different ZG, which coincided with the known AG and subgroups (Figure 2).

Discussion

ITS rDNA polymorphism within AG 2

For the development of control measures of bare patch in field-grown flower bulbs based on the ecology of the pathogens, unambiguous identification of the causal AG 2-t isolates is essential. *R. solani* AG 2 is the only AG that is subdivided by hyphal fusion frequency. Due to great variation in hyphal fusion frequency, each of AG 2-t isolates cannot be distinguished from Dutch AG 2-1 isolates non-pathogenic to tulips (Schneider et al., 1997). PCR showed small differences in ITS rDNA fragment size between AG 2-t isolates, pathogenic to crucifers and tulips, and Dutch AG 2-1 isolates, non-pathogenic to tulips (Tables 3 and 4). Hence, PCR of ITS rDNA supports previous results on hyphal fusion frequency and pathogenicity to assign AG 2 isolates infecting tulips to the subgroup of AG 2-t (Schneider et al., 1997). In our ITS-PCR experiments, AG 2-t isolates could not be distinguished from the Japanese anastomosis tester isolate (21R01) (Table 3; Figure 1), whereas Dutch AG 2-1 isolates differed slightly in ITS rDNA fragment length from the 21R01 isolate. Kanematsu and Naito (1995) reported their AG 2-3 isolates to have the same ITS rDNA fragment length (690 bp) as their AG 2-1 isolates. Our limited set of AG 2-t isolates displayed a high degree of ITS rDNA polymorphism and we anticipate more variation when screening numerous field isolates. Such variation may be useful for the study of the population structure of the pathogen but not for routine identification procedures.

In the analysis of ITS rDNA polymorphism some AG 2-1 and AG 2-t isolates showed heterogeneous fragment digestion patterns using restriction endonuclease *Hinc* II (Table 4; Figure 1). The heterogeneous fragment digestion pattern seemed to consist of equal ratios of two patterns. Neither the use of additional enzyme, nor the use of extended incubation periods changed the digestion pattern, thus excluding partial digestion. Contamination was excluded since independent DNA isolations from pure cultures, that were repurified several times by hyphal tip transfer over WA with antibiotics (250 ppm chloramphenicol), by two individuals gave the same results. The heteroge-

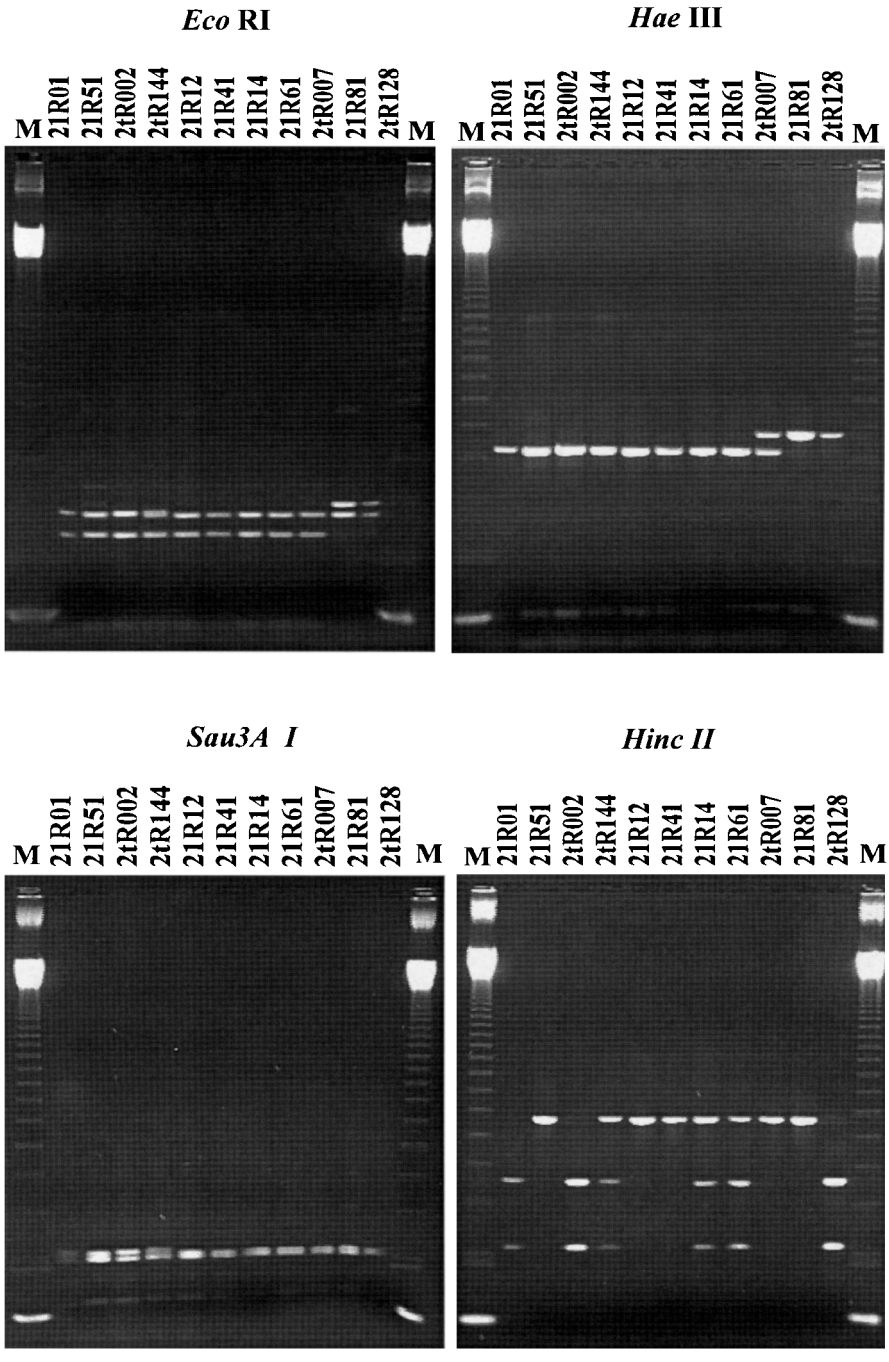

Figure 1. Digestion pattern of ITS rDNA regions of Dutch AG 2-t isolates (2tR002, 2tR007, 2tR128 and 2tR144), Dutch AG 2-1 isolates (21R12, 21R14, 21R41, 21R51, 21R61 and 21R81) and 1 Japanese isolate (21R01) after digestion with 4 endo-nucleases. M: 123 bp molecular marker.

neous fragment digestion pattern occurred only with *Hinc* II and not with other endonucleases. Heterogeneous fragment digestion patterns were not reported previously for *R. solani*, but different ITS sequences within an individual isolate were reported for *R. solani* AG 4 (Boysen et al., 1996). Different ITS sequences

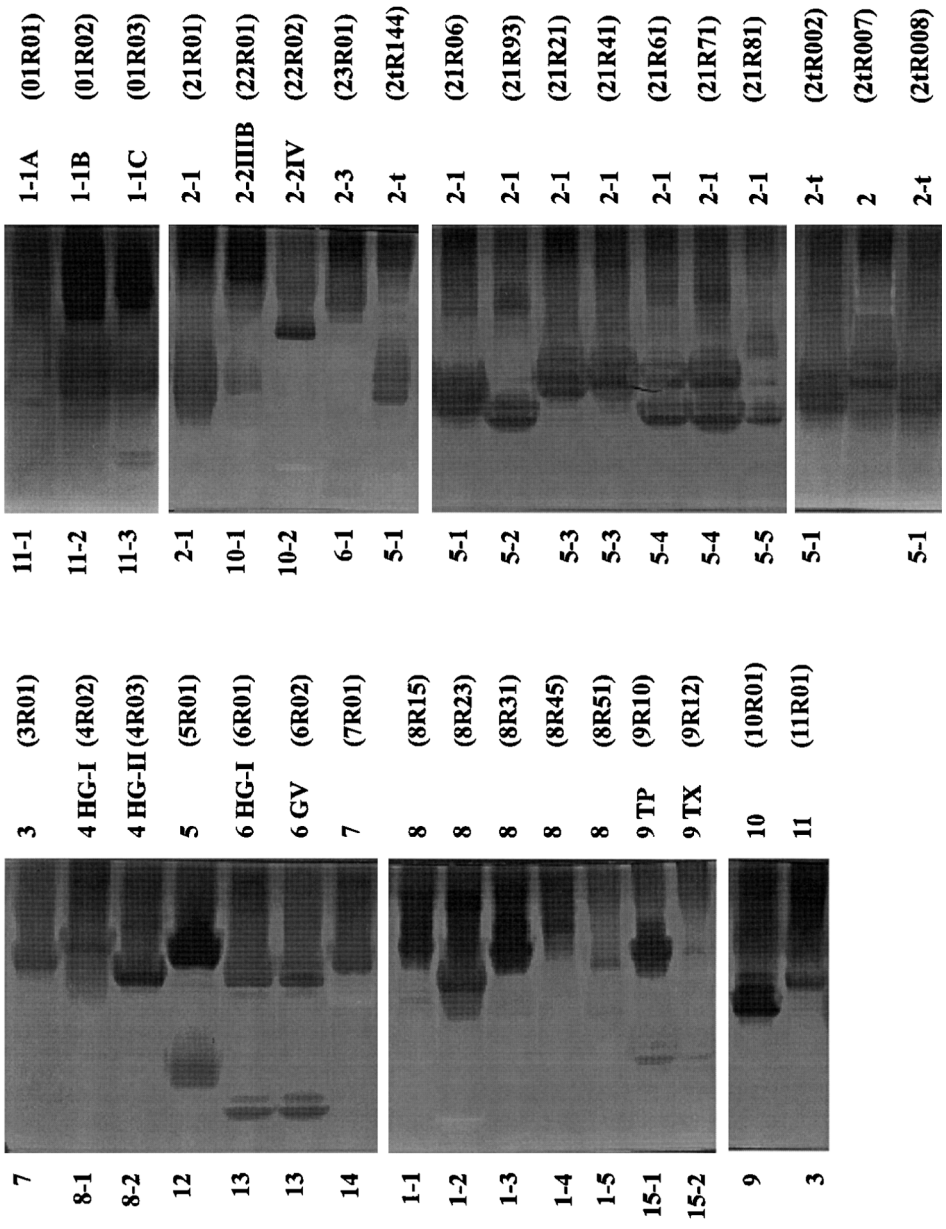

Figure 2. Pectic zymograms of *Rhizoctonia solani* anastomosis tester isolates, some AG 2-1 and AG 2-t isolates and isolate 2tR007 obtained with a commercial available vertical gel-electrophoresis system. The AG is displayed on top and the ZG on the bottom of the gels. Isolate 2tR007 was unassigned to zymogram groups.

within a single individual also have been reported for the nematode *Meloidogyne hapla* (Zijlstra et al., 1995). The three different heterogeneous digestion patterns that can be distinguished within *R. solani* AG 2 seem to appear at random and are not correlated with origin or pathogenicity. The occurrence of heterogeneous fragment digestion patterns can be explained by the heterokaryotic nature of *R. solani*, with one nucleus differing slightly from the other. It may indicate a step in evolutionary divergence. Whether this heterokaryotic nature originates from 'bridging phenomena' between isolates of different subgroups or is the result of genetic variation triggered by the teleomorph is unknown and requires further study. Another explanantion may be that different chromosomes comprise different ITS sequences.

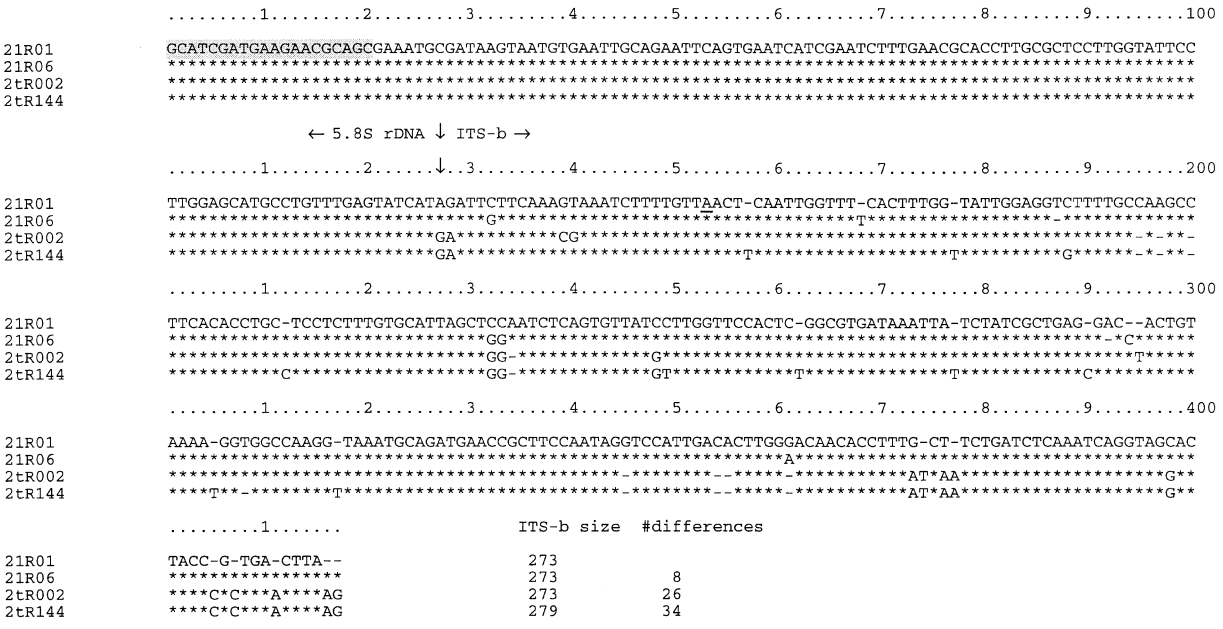

Figure 3. Aligment of nucleotides of the ITS-b region (starting at ↓) of two Japanese AG 2-1 isolates (21R01 and 21R06) and two AG 2-t isolates (2tR002 and 2tR144). Highlighted is the ITS-3 primer site on the 5.8S ribosomal subunit of which 126 nucleotides (total = 155) are shown. An asterisk indicates the same nucleotide and a dash (–) a deletion in comparison with the 21R01 isolate. A dash in the 21R01 isolate indicates an insertion in one or more of the other isolates. ITS-b size: number of nucleotides in the ITS-b region per isolate. #differences: number of different nucleotides in comparison with the 21R01 isolate. The A indicates the *Hinc* II restriction site.

Identification of AG-2 isolates by means of pectic zymograms

Pectic zymography has been successfully used for the identification of numerous field populations of *R. solani* (Neate et al., 1988; Sweetingham, 1990; MacNish et al., 1994). They used a non-commercial available horizontal poly-acrylamide gel system (Cruickshank and Wade, 1980). We made use of commercially available vertical poly-acrylamide gel system, thus making the zymogram grouping method accessible outside Australia. Using our ZG technique, AG 2-t isolates could easily be distinguished from AG 2-1 isolates which were non-pathogenic to tulips (Figure 2). *R. solani* AG 2-t isolates belonged to a homogeneous ZG that also included AG 2-1 isolates 21R01 and 21R06 originating from Japan (Figure 2) and isolate 21R93 from Alaska. Isolates 21R01 and 21R06 were pathogenic to tulips in greenhouse experiments (Schneider, unpubl.). The pathogenicity of isolate 21R93 has not been tested. ZG-typing is in agreement with our ITS rDNA results and show that our adapted pectic zymography is a reliable identification method for bare patch causing isolates of *R. solani*. The method can be used for research on *Rhizoctonia* populations and their dynamics e.g. in field experiments aimed at the development of environmentally friendly control measures of rhizoctonia disease in flower bulb production.

AG 2 isolates pathogenic to crucifers and flower bulbs constitute a distinct ZG within AG 2. In previous studies AG 2-1 isolates pathogenic to crucifers and legumes were shown to belong to ZG5 and ZG6, respectively (Sweetingham, 1990; MacNish et al., 1994). In addition, Cruickshank (1990) reported two isolates (21R01 and 21R06; Table 1) with the general characteristics of ZG5 which also were pathogenic to crucifers and tulips (Schneider et al., 1997; Schneider unpubl.). For these reasons and for consistency with the ZG-typing within AG 8, we propose to assign *R. solani* AG 2 isolates, affecting crucifers and tulips, to ZG5-1. Dutch *R. solani* AG 2-1 isolates affecting crucifers but not tulips gave different ZG patterns (Table 2, Figure 2). Three soybean leaf infecting AG 2-3 isolates (Naito and Kanematsu, 1994) gave a homogeneous ZG different from other AG 2 isolates. AG 2-1 isolates affecting legumes and barley in Australia show a ZG6 pattern (Sweetingham, 1990; MacNish et al., 1994). Since ZG6 isolates were not included in our study, their relation to AG 2-3 infecting soybean remains unclear. We propose to assign AG 2-3 isolates to ZG6-1.

R. solani isolate 2tR007 was confirmed to belong to a ZG other than isolates 21R01 and 21R06. Isolate 2tR007, isolated from cold glasshouse-grown tulips in the Netherlands, was previously designated as AG 2-3 (Cruickshank, 1990). Cruickshank also described isolate 2tR007 and two *R. solani* isolates obtained from *Brassica oleracea* in New Zealand, to have the same ZG pattern, but different from 21R01 and 21R06 in his study. Cruickshank (1990), however, did not assign any of the isolates to a ZG. In our ZG screening of isolates obtained from tulips, isolate 2tR007 gave a ZG-pattern different from all other isolates. Our ITS rDNA digestion pattern confirmed the genetic relation of isolate 2tR007 to AG 2 isolates. The pathogicity of isolate 2tR007 to tulips has not been tested, and therefore it remains unclear whether this isolate belongs to AG 2-t.

Identification of subgroups in AG 2 and AG 4

The distinction of AG 2-1 and AG 2-2 on the basis of low hyphal fusion frequency coincides with marked differences in DNA base homology (Kuninaga and Yokosawa, 1982) and ITS rDNA fragment lengths and polymorphism (Liu et al., 1992) (Table 3), pectic zymography (Figure 2), cultural characteristics, and host range (Ogoshi, 1987). In addition, it has been observed that low-frequency fusion, bridging, can occur between AG 2 isolates and isolates belonging to AG 3, AG 8, AG 11 and AG BI (Kuninaga et al., 1979; Neate et al., 1988; Carling et al., 1994; Carling, 1996). Therefore, it is questionable whether hyphal fusion frequency remains a reliable criterion to determine subgroups within AG 2 and whether AG 2-2 still has to be regarded as a subgroup of AG 2, rather than a distinct AG.

The clear distinction of biologically relevant subgroups within AG 2-1 using pectic zymography supports the suggestion of MacNish et al. (1994) to use the ZG system for subgrouping within AG, especially since ZG do not seem to cross AG boundaries. ZG typing, both more practical and clearer than the use of hyphal fusion frequency, may be used as an alternative for other subgrouping techniques e.g. DNA homology or host range. An overview of zymogram groups distinguished in this paper is given in Table 2 and Figure 2. AG 2-2 is subdivided into AG 2-2IIIB and AG 2-2IV on the basis of pathogenicity. This division is supported by DNA base sequence homology (Kuninaga and Yokosawa, 1982) and ITS rDNA polymorphism (Liu et al., 1992; Table 3). Indeed, these two groups represent distinct ZG (Figure 2). In addition, one other ZG-pattern within AG 2-2 could be identified using our pectic zymography (Table 2). Within AG 4, subgroups AG 4 HG-I and AG 4 HG-II are distinguished on the basis of DNA-homology (Kuninaga and Yokosawa, 1984). This division was supported by our ITS rDNA polymorphism (Table 4). The HG-I and II subgroups were shown to belong to different ZG (Figure 2).

R. solani AG 2 isolates affecting both tulips and crucifers had the same ZG, regardless of their geographic origin. Sequence analysis revealed substantial differences between two AG 2-t and two Japanese AG 2-1 isolates. It is unclear whether AG 2 populations with the same ZG originated from the same gene centre or evolved independently at different geographical sites. Sequence data and phylogeny of AG 2-t and AG 2 isolates from different geographical areas are to be presented elsewhere (Salazar et al., unpubl.).

Our data underline the observations of Sweetingham et al. (1986) that ZG may be related to pathogenicity. However, conclusive experiments relating ZG to pathogenicity are lacking. Pectic zymograms comprise the patterns of different isozymes of polygalacturonase and pectin esterase (Cruickshank and Wade, 1980) and are supposed to reflect different genes. Polygalacturonase is a major enzyme in tissue maceration caused by *R. solani* (Bateman, 1963), produced in response to host exudates (Brookhouser and Weinhold, 1979) and related to virulence (Geypens, 1978). To justify the subgrouping of ZG within AG to pathogenicity and disease requires characterization of genes encoding different isozymes of polygalacturonase and subsequent confirmation of the role of polygalacturonase in pathogenesis. The clearance of such genes may provide molecular markers for the detection of host related *R. solani* isolates in plant material and in soil and the study of the dynamics of specific isolates in space and time.

Acknowledgements

Part of this work was financially supported by the Urgency Program for Research on Diseases and Breeding of Flower Bulbs. The authors thank the Bulb Research Centre (LBO), the Plant Protection Service (PD), Drs D.E. Carling, G. Jager, A.P. Keinath, S. Naito, G.C. MacNish, and A. Ogoshi, for supplying isolates of *R. solani*. M.J.M. Wagemaker is thanked for skilled technical assistance. Drs N.J. Fokkema

and J.C. Zadoks are gratefully acknowledged for valuable comments and Dr S.M. Neate for making English corrections.

References

Bateman DF (1963) The "Macerating Enzyme" of *Rhizoctonia solani*. Phytopathology 53: 1178–1186

Boysen M, Borja M, Del Moral C, Salazar O and Rubio V (1996) Identification at strain level of *Rhizoctonia solani* AG 4 isolates by direct sequencing of asymmetric PCR products of the ITS regions. Current Genetics 29: 174–181

Brookhouser LW and Weinhold AR (1979) Induction of polygalacturonase from *Rhizoctonia solani* by cotton seed and hypocotyl exudates. Phytopathology 69: 599–602

Carling DE (1996) Grouping in *Rhizoctonia solani* by hyphal anastomosis reaction. In: Sneh B, Jabaji-Hare S, Neate SM and Dijst G (eds) *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. pp. 37–47. Dordrecht: Kluwer

Carling DE and Sumner DR (1992) *Rhizoctonia*. In: Singleton LL, Mihail JD and Rush (eds) Methods for Research on Soilborne Phytopathogenic Fungi. St.Paul, Minnesota: APS

Carling DE, Rothrock CS, MacNish GC, Sweetingham MW, Brainard KA and Winters SW (1994) Characterization of Anastomosis Group 11 (AG-11) of *Rhizoctonia solani*. Phytopathology 84: 1387–1393

Cruickshank RH (1990) Pectic zymograms as criteria in taxonomy of *Rhizoctonia*. Mycological Research 94: 938–946

Cruickshank RH and Wade GC (1980) Detection of pectic enzymes in pectin-acrylamide gels. Analytical Biochemistry 107: 177–181

Doornik AW (1981) Temperature dependence of the pathogenicity of several isolates of *Rhizoctonia solani* in some bulb crops as an intrinsic property of the isolate. Netherlands Journal of Plant Pathology 87: 139–147

Duncan S, Barton JE and O'Brien PA (1993) Analysis of variation in isolates of *Rhizoctonia solani* by random amplified polymorphic DNA assay. Mycological Research 97: 1075–1082

Gardes M and Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113–118

Geypens M (1978) Enzymatic production and virulence of *Rhizoctonia solani* isolates. Annals of Phytopathology 3: 355–363

Gyllensten UB and Erlich HA (1988) Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proceedings of the National Academy of Science USA 85: 7652–7656

Innis MA and Gelfand DH (1990) Optimization of PCRs. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds) PCR Protocols, A Guide to Methods and Applications (pp 3–12). San Diego, CA: Academic Press

Jabaji-Hare SH, Meller Y, Gill S and Charest PM (1990) Investigation of genetic relatedness among anastomosis groups *Rhizoctonia solani* using cloned DNA probes. Canadian Journal of Plant Pathology 12: 393–404

Kanematsu S and Naito S (1995) Genetic characterization of *Rhizoctonia solani* AG-2-3 by analyzing restriction fragment length polymorphisms of nuclear ribosomal DNA internal transcribed spacers. Annals of the Phytopathological Society of Japan 61: 18–21

Keijer J, Houterman PM, Dullemans AM and Korsman MG (1996) Heterogeneity in electrophoretic karyotype within and between anastomosis groups of *Rhizoctonia solani*. Mycological Research 100: 789–797

Kuninaga S and Yokosawa R (1982) DNA base sequence homology in *Rhizoctonia solani* Kühn. II. Genetic relatedness within anastomosis group 2. Annals of the Phytopathological Society of Japan 48: 668–673

Kuninaga S and Yokosawa R (1984) DNA base sequence homology in *Rhizoctonia solani* Kühn. IV. Genetic relatedness within AG-4. Annals of the Phytopathological Society of Japan 50: 322–330

Kuninaga S, Yokosawa R, and Ogoshi A (1979) Some properties of anastomosis group 6 and BI in *Rhizoctonia solani* Kühn. Annals of the Phytopathological Society of Japan 45: 207–217

Laemmli UK (1970) Cleavage of the structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685

Liu ZL and Sinclair JB (1992) Genetic diversity of *Rhizoctonia solani* Anastomosis Group 2. Phytopathology 82: 778–787

Liu ZL, Domier LL and Sinclair JB (1993) ISG-specific ribosomal DNA polymorphism of the *Rhizoctonia solani* species complex. Mycologia 85: 795–800

Liu ZL, Domier LL and Sinclair JB (1995) Polymorphism of genes coding for nuclear 18S rRNA indicates genetic distinctiveness of anastomosis group 10 from other groups in the *Rhizoctonia solani* species complex. Applied and Environmental Microbiology 61: 2659–2664

Liu ZL and Sinclair JB (1993) Differentiation of intraspecific groups within anastomosis group 1 of *Rhizoctonia solani* using ribosomal DNA internal transcribed spacer and isozyme comparisons. Canadian Journal of Plant Pathology 15: 272–280

MacNish GC and Sweetingham MW (1993) Evidence of stability of pectic zymogram groups within *Rhizoctonia solani* AG-8. Mycological Research 97: 1056–1058

MacNish GC, Carling DE, Sweetingham MW and Brainard KA (1994) Anastomosis group (AG) affinity of pectic isozyme (zymogram) groups (ZG) of *Rhizoctonia solani* from the Western Australian cereal-belt. Mycological Research 98: 1369–1375

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. Annals of the Phytopathological Society of Japan 60: 681–690

Neate SM, Cruickshank RH and Rovira AD (1988) Pectic enzyme patterns of *Rhizoctonia solani* isolates from agricultural soils in South Australia. Transactions of the British Mycological Society 90: 37–42

O'Brien PA (1994) Molecular markers in Australian isolates of *Rhizoctonia solani*. Mycological Research 98: 665–671

Ogoshi A (1975) Grouping of *Rhizoctonia solani* Kühn and their perfect stages. Review of Plant Protection Research 8: 93–103

Ogoshi A (1987) Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annual Review of Phytopathology 25: 125–143

Parmeter JR (ed) (1970) *Rhizoctonia solani*: Biology and Pathology. Berkeley, CA, USA: University of California Press

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Science USA 74: 5463–5467

Schneider JHM, Schilder MT and Dijst G (1997) Characterization of *Rhizoctonia solani* AG-2 in flower bulbs in the Netherlands. European Journal of Plant Pathology 103: 265–279

Sneh B, Burpee L and Ogoshi A (1991) Identification of *Rhizoctonia* species. St Paul, Minnesota: APS

Sneh B, Jabaji-Hare S, Neate, SM and Dijst G (eds) (1996) *Rhizoctonia* species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Dordrecht: Kluwer

Stevens Johnk J, Jones RK, Shew HD and Carling DE (1993) Characterization of populations of *Rhizoctonia solani* AG-3 from potato and tobacco. Phytopathology 83: 854–858

Sweetingham MW (1990) Coping with brown spot and root rots of lupins. The Journal of Agriculture, Western Australia 31: 5–13

Sweetingham MW, Cruickshank RH and Wong DH (1986) Pectic zymograms and taxonomy and pathogenicity of the *Ceratobasidiaceae*. Transactions of the British Mycological Society 86: 305–311

Van den Boogert PHJF and Jager G (1984) Biological control of *Rhizoctonia solani* on potatoes by antagonists. 3. Inoculation of seed potatoes with different fungi. Netherlands Journal of Plant Pathology 90: 117–126

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

White TJ, Bruns T, Lee S and Taylor J, (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds) PCR Protocols, A Guide to Methods and Applications (pp 315–322). San Diego, CA: Academic Press

Yang HA, Sivasithamparam K, Barton JE and O'Brien PA (1995) Characterization of cereal bare patch isolates of *Rhizoctonia solani* by random amplified polymorphic DNA analysis. Plant Pathology 44: 811–818

Zijlstra C, Lever AEM, Uenk BJ and Van Silfhout CH (1995) Differences between ITS regions of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. Phytopathology 85: 1231–1237