Eur J Plant Pathol (2007) 117:247–265
DOI 10.1007/s10658-006-9091-7

FULL RESEARCH PAPER

Genetic diversity, anastomosis groups and virulence of *Rhizoctonia* spp. from strawberry

Michal Sharon · Stanley Freeman · Shiro Kuninaga · Baruch Sneh

Received: 17 July 2006 / Accepted: 18 December 2006 / Published online: 23 January 2007

Abstract Virulent *Rhizoctonia* spp. isolated from strawberry in Israel belonged to anastomosis groups (AG) of: binucleate *Rhizoctonia* (BNR) AG-A, AG-G, AG-K and AG-F, and to multinucleate *Rhizoctonia* (MNR) AG 4 subgroup HG-I. In addition, a soil isolate of AG 4 subgroup HG-III was also found to be virulent on strawberry. None of the Israeli isolates obtained in the present study belonged to BNR AG-I, or other MNR AGs. In the cluster analysis of rDNA-ITS sequences, all of the isolate sequences consistently clustered according to their known AGs and subgroups. One AG-F cluster included sequences of 10 strawberry isolates, while another AG-F cluster included sequences of two isolates submitted to GenBank. Additional work is needed to determine whether the isolates of these two clusters may belong to different AG-F subgroups. The current virulence bioassay used for *Rhizoctonia* spp. isolates on strawberry is based on inoculation of stolon-derived daughter plants with the isolates and estimation of the reduction in plant biomass, rather than on specific distinct disease severity symptoms. The duration of this test is relatively long (ca. 5 weeks or more) and the availability of daughter plants from runners is naturally limited to a certain season. Among the possible alternative methods evaluated in the present study (inoculation of fruits or seedlings developed from germinated strawberry seeds), the method based on seedlings was best. This method has a potential to replace the currently used stolon-daughter plant inoculation bioassay for testing virulence of strawberry root pathogens. This is the first report indicating that *Rhizoctonia* spp. isolates that belong to AG-F, AG-K, AG 4 HG-I and AG 4 HG-III are virulent to strawberry.

Keywords Black root rot · Daughter plants · *Fragaria ananassa* · Hard rot · Percent sequence similarity · rDNA-ITS sequence analysis · *Rhizoctonia praticola* · Virulence tests

Abbreviations

AG	Anastomosis group
ANOVA	Analysis of variance
ap-PCR	Arbitrarily primed polymerase chain reaction

M. Sharon · B. Sneh (✉)
Department of Plant Sciences, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel
e-mail: baruchs@tauex.tau.ac.il

S. Freeman
Department of Plant Pathology, Agriculture Research Organization, The Volcani Center, Bet Dagan 50250, Israel

S. Kuninaga
Department of Integrated Human Sciences, School of Dentistry, Health Sciences University of Hokkaido, Tohbetsu, Hokkaido 061-0293, Japan

BNR	binucleate *Rhizoctonia*
DDBJ	DNA data bank of Japan
MNR	Multinucleate *Rhizoctonia*
np-R	Non-pathogenic or hypovirulent *Rhizoctonia*
rDNA-ITS	Ribosomal DNA-internal transcribed spacer
RFLP	Restriction fragment length polymorphism
Str	Strawberry isolates

Introduction

Binucleate *Rhizoctonia fragariae* (BNR) and multinucleate *R. solani* (MNR) are among the major pathogens causing the strawberry (*Fragaria ananassa*) black root rot complex. These pathogens also infect other parts of the strawberry plant, causing stunting, necrosis, yield reduction and, occasionally, plant death (Hancock, 1999). The pathogens are mainly distributed by infected propagation materials, originating from mother plants via stolon production of daughter plants (Maas, 1984). Soil inoculum is also an important source for infection of strawberry plants. Once the plants are infected, the melanized hyphae and sclerotia of the pathogen are surrounded by sloughed plant cells, thus improving their survival rate in soil (Maas, 1984). Subsequently, these propagules are dispersed and infect roots of the same and adjacent plants (Maas, 1984; Wilhelm, Nelson, Thomas, & Johnson, 1972). Since the strawberry root system is dense and not widespread, the whole root system eventually becomes severely infected (Hancock, 1999). *Rhizoctonia* spp. also infect fruit, buds and leaves, causing hard rot of fruit and foliar blight, respectively (Maas, 1984).

The most common and classical nomenclature for *Rhizoctonia* spp. isolates is based on the anastomosis group (AG) concept (Carling, 1996; Sneh, Burpee, Ogoshi, 1991). Strawberry plants were reported to be infected by *R. fragariae* isolates belonging to AG-A, AG-G and AG-I of BNR species (Sneh et al., 1991) in California (Martin, 2000), in Connecticut (Martin, 1998) and in South Africa (Botha, Denman, Lamprecht, Mazzola, & Crous, 2003). In Connecticut, strawberry plants were infected also by *R. solani* AG-5 (MNR), but not severely (Martin, 1988), while they were severely infected by virulent *R. solani* AG-6 in South Africa (Botha et al., 2003). No detailed studies have been previously reported on Rhizoctonia diseases of strawberry in Israel.

Various biochemical and molecular methods have been used to classify *Rhizoctonia* spp. isolates. Isozyme analyses identified isolates to their AGs and subgroups (Banniza & Rutherford, 2001; Damaji, Jabaji-Hare, & Charest, 1993; Laroche, Jabaji-Hare, & Charest, 1992; Liu & Sinclair, 1992). Specific molecular markers or primers were developed to identify BNR isolates belonging to BNR AG-G (Leclerc-Potvin, Balmas, Charest, & Jabaji-Hare, 1999) and MNR *R. solani* AG 2 subgroups (Salazar, Julian, & Rubio, 2000). A cladogram, based on RFLPs of a portion of the 28S rDNA was used to cluster isolates of *Rhizoctonia* spp. from strawberry (Martin, 2000). Nevertheless, the use of rDNA-ITS sequence analyses was superior in clustering tested isolate sequences to groups concurrent with their AGs and subgroups (Carling, Kuninaga, & Brainard, 2002), but was not previously used for strawberry isolates. Although the molecular methods may be more accurate, handy and less time consuming, sometimes, they need to be verified by the classical anastomosis grouping method.

The currently used virulence test for *Rhizoctonia* spp. in strawberry is based on inoculating stolon-derived strawberry daughter plants (Martin, 1988, 2000; Wilhelm et al., 1972) or tissue culture-derived plants (Botha et al., 2003). In this method, disease severity caused by a *Rhizoctonia* spp. isolate is evaluated by comparing the fresh and/or dry weight of the inoculated plants with non-inoculated control plants after 3–5 weeks (Botha et al., 2003; Martin, 1988, Wilhelm et al., 1972), or up to a whole year of growth (Wilhelm et al., 1972) as the disease in inoculated plants may only become apparent later in the season. Since plant biomass may also be affected by various additional environmental factors such as light, temperature, humidity, initial plant vigour, etc., this method may therefore not be sufficiently reliable. This method is also time-consuming, limited to the

season of stolon development, availability and variability of the new daughter plants.

The hypothesis of the present work is to determine whether *Rhizoctonia* spp. isolates recovered from infected strawberry plants collected from fields and nurseries in Israel are of the same AGs as those found in other countries, or there are also isolates of additional AGs and whether classification with the rDNA-ITS sequence analysis of the strawberry isolates corresponds with the classical method of anastomosis grouping. Attempts were made to develop an alternative and more reliable method for determining virulence of *Rhizoctonia* spp. isolates on strawberries, based on typical disease symptoms, independent of the season and less time-consuming than the currently used method using the stolon-derived plants.

Materials and methods

Rhizoctonia spp. isolates

Isolations of *Rhizoctonia* spp. were made from roots or crowns of different cultivars of strawberry plants with brown necrotic symptoms collected arbitrarily throughout the growing season from 2000 to 2003, from various fields and nurseries in Israel (Sharon region). Infected roots, crowns and petioles were washed in running tap water, surface disinfested in 1% sodium hypochlorite (NaOCl) solution for 1 min, washed twice in sterile water, blotted on paper towels and plated on water agar supplemented with 250 μg ml^{-1} chloramphenicol (WAcm). Emerging hyphal tips were transferred to potato dextrose agar plates supplemented with 250 μg ml^{-1} chloramphenicol (PDAcm) as described previously (Ichielevich-Auster, Sneh, Barash, & Koltin, 1985). These isolates were designated as Str#. In addition, representative virulent strains from strawberry (Am1, Am2, Gm1, Gm2, Im1, and Im2) were obtained from F.N. Martin (USDA, ARS, Salinas, California), and rDNA-ITS sequences of representative isolates of different AGs of *Rhizoctonia* spp. were retrieved from GenBank as summarized in Table 1. Air-dried wheat grains colonized with the isolates were used for culture preservation and for inoculum, and were prepared as described previously (Sneh & Adams, 1996; Sneh, Zeidan, Ichielevich-Auster, Barash, & Koltin, 1986).

Anastomosis groups (AGs) of *Rhizoctonia* spp. isolates from strawberry

The procedure for determining anastomosis (hyphal fusion) was carried out on cellophane sheet disks and aseptically placed on WAcm plates, as described previously (Carling, 1996; Martin, 2000). Anastomosis of >50% of the meetings between the examined and the tester isolate hyphal tips indicated that they belong to the same AG or subgroup, while anastomosis of <50% were typical for anastomosis among subgroups or some AGs, which do anastomose (Sneh et al., 1991). The following tester isolates were used to identify the belonging to the corresponding AGs: SN-2, TKR3, AM1 (for AG-A); C-314 (AG-Ba); MAFF305285 (AG-Bb); C-302 [AG-B(o)]; OR706 (AG-C); *R. cerealis* (AG-D); MAFF 305296, ...98, ...99, TMA1-1 (AG-E); FKO2-28, PS-17, SIR-1, NP2011 (AG-F); Hak1-1, NP2007, TKE1, Gm2 (AG-G); STC-9 (AG-H); AV-2 (AG-I); AC-1 (AG-K); Bn-37 (AG-R); AH-1 (AG 4 HG-I); RH-165 (AG 4 HG-II); BR-2 (AG 4 HG-III).

DNA extraction

The mycelia of *Rhizoctonia* isolates grown on potato dextrose broth supplemented with 250 μg ml^{-1} chloramphenicol (PDBcm) for 10 days were collected by vacuum filtration and lyophilized to dryness. DNA was extracted and purified as described by Freeman, Pham, and Rodriguez (1993), the DNA was suspended in 0.5 ml of TE buffer, diluted 1:10 in distilled H_2O and stored at 4°C until use.

rDNA-ITS sequencing

The rDNA-ITS fragment (ribosomal ITS1, ITS2 and 5.8S gene) was amplified using primers: ITS4- (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5-(5′-GGAAGTAAAAGTCGTAACA-AGG-3′) (White, Bruns, Lee, & Taylor, 1990).

Table 1 *Rhizoctonia* spp. isolates classification, origin and virulence, as determined by two virulence test methods

Isolate #	Accession	Anastomosis groups	Isolate origin	Virulence, %[a]		
				Fruit	Seedlings	
				25°C	25°C	30°C
None	–	–	–	0	5	3
Rs13	DQ102445	4 HG-I	Soil, Israel[b1]	100*	75*	60*
Str43[c]	DQ102446	4 HG-I	Strawberry, Israel[b1]	25	8	23
Str44	DQ102447	4 HG-I	Strawberry, Israel[b1]	83*	48*	43*
Str45	DQ102448	4 HG-I	Strawberry, Israel[b1]	58*	58*	55*
TO-4	AY152704	4 HG-I	Unknown[b2]	N[d]	N	N
AH-1	AY154307	4 HG-I	Unknown[b2]	N	N	N
UHBC	AB000045	4 HG-II	Sugar beet, Japan[b3]	N	N	N
Rh-264	AB000034	4 HG-II	Sugar beet, Japan[b3]	N	N	N
Rs20	DQ102449	4 HG-III	Soil, Israel[b1]	40*	3	5
EP-3	AY154660	4 HG-III	Unknown[b2]	N	N	N
BR-2	AY152812	4 HG-III	Unknown[b2]	N	N	N
Am1	DQ102403	A	Strawberry, USA[b1]	91*	83*	28*
Str3	DQ102421	A	Strawberry, Israel[b1]	35*	8	0
Str4	DQ102406	A	Strawberry, Israel[b1]	14	8	3
Str5	DQ102410	A	Strawberry, Israel[b1]	34*	3	10
Str6	DQ102407	A	Strawberry, Israel[b1]	9	3	8
Str7	DQ102408	A	Strawberry, Israel[b1]	11	0	20
Str8	DQ102422	A	Strawberry, Israel[b1]	42*	13	15
Str9	DQ102428	A	Strawberry, Israel[b1]	25	8	33*
Str11	DQ102411	A	Strawberry, Israel[b1]	28	28	10
Str12	DQ102409	A	Strawberry, Israel[b1]	39*	3	3
Str21	DQ102415	A	Strawberry, Israel[b1]	0	5	15
Str22	DQ102423	A	Strawberry, Israel[b1]	34*	10	23
Str23	DQ102424	A	Strawberry, Israel[b1]	11	10	18
Str25	DQ102419	A	Strawberry, Israel[b1]	0	18	10
Str28	DQ102405	A	Strawberry, Israel[b1]	45*	30	33*
Str34	DQ102425	A	Strawberry, Israel[b1]	50*	13	8
Str38	DQ102427	A	Strawberry, Israel[b1]	25	0	48*
Str42	DQ102416	A	Strawberry, Israel[b1]	58*	20	13
Str75	DQ102426	A	Strawberry, Israel[b1]	25	0	3
Str77	DQ102418	A	Strawberry, Israel[b1]	0	8	15
Str78	DQ102404	A	Strawberry, Israel[b1]	19	23	5
Str81	DQ102412	A	Strawberry, Israel[b1]	25	0	30*
Str96	DQ102420	A	Strawberry, Israel[b1]	46*	3	13
Str116	DQ102413	A	Strawberry, Israel[b1]	30	5	5
RH521	U19950	A	Soil, Israel[b5]	N	N	N
RU56-8	DQ102417	A	Soil, USA[b1]	N	N	N
SIR-2[f]	AF354091	A	Sweet potato, Japan[b6]	N	N	N
Am2	DQ102414	A	Strawberry, USA[b1]	N	N	N
SN-2	AB000040	A	Soil Japan[b3]	N	N	N
C-460	AF354088	Ba	Rice, Japan[b6]	N	N	N
C-350	AB122144	Bb	Rice, Japan[b7]	N	N	N
C1	AJ000192	Bb	Unknown[b8]	N	N	N
C6	AJ000194	Bb	Unknown[b8]	N	N	N
RU18-1	DQ102430	B(o)	Soil, USA[b1]	0	10	5
RU89-1	DQ102431	B(o)	Soil, USA[b1]	30	3	0
C-302	AB219143	B(o)	Soil, Japan[b4]	N	N	N
Str10	DQ102434	F	Strawberry, Israel[b1]	0	3	18
Str36	DQ102435	F	Strawberry, Israel[b1]	55*	25	20
Str46	DQ102440	F	Strawberry, Israel[b1]	30	5	3
Str47	DQ102441	F	Strawberry, Israel[b1]	0	28	23
Str51	DQ102437	F	Strawberry, Israel[b1]	0	10	25
Str52	DQ102436	F	Strawberry, Israel[b1]	3	8	43*

Table 1 continued

Isolate #	Accession	Anastomosis groups	Isolate origin	Virulence, %[a]		
				Fruit	Seedlings	
				25°C	25°C	30°C
Str55	DQ102439	F	Strawberry, Israel[b1]	38*	13	15
Str56	DQ102438	F	Strawberry, Israel[b1]	0	5	13
Str110	DQ102432	F	Strawberry, Israel[b1]	9	0	25
Str111	DQ102433	F	Strawberry, Israel[b1]	3	0	23
Ps-17	AB219144	F	Pea, Japan[b4]	N	N	N
SIR-1	AF354085	F	Sweet potato, Japan[b6]	N	N	N
FKO2-28	AB219145	F	Soil, Japan[b4]	N	N	N
BN-38	AF354081	F	Soybean, USA[b6]	N	N	N
BN-37gb[g]	AF354082	F	Cucumber, USA[b6]	N	N	N
Gm1	DQ102395	G	Strawberry, USA[b1]	35*	55*	33*
Gm2	DQ102397	G	Strawberry, USA[b1]	N	N	N
Str13	DQ102398	G	Strawberry, Israel[b1]	7	63*	10
Str14	DQ102402	G	Strawberry, Israel[b1]	3	73*	15
Str15	DQ102396	G	Strawberry, Israel[b1]	7	8	5
Str16	DQ102401	G	Strawberry, Israel[b1]	31*	55*	8
Str31	DQ102399	G	Strawberry, Israel[b1]	39*	8	8
Str35	DQ102400	G	Strawberry, Israel[b1]	50*	13	40*
Im1	DQ102443	I	Strawberry, USA[b1]	25	30	3
Im2	DQ102444	I	Strawberry, USA[b1]	N	N	N
Ibs1	DQ102442	I	Soil[b1]	17	0	5
AV-2	AJ419932	I	Artemisia, Japan[b9]	N	N	N
Str24	DQ102429	K	Strawberry, Israel[b1]	10	5	28*
AC-1	AB122145	K	Onion, Japan[b7]	N	N	N
FA59209	AJ242900	K	Unknown[b10]	N	N	N
Bn37	AB219146	R	Cucumber USA[b4]	N	N	N
AV-2(r)[h]	AJ242898	Unknown	Unknown[b11]	N	N	N
Str17	N.D.[e]	N.D.	Strawberry, Israel	52*	28	23
Str18	N.D.	N.D.	Strawberry, Israel	3	15	15
Str19	N.D.	N.D.	Strawberry, Israel	23	0	5
Str26	N.D.	N.D.	Strawberry, Israel	20	3	8
Str30	N.D.	N.D.	Strawberry, Israel	77*	8	75*
Str32	N.D.	N.D.	Strawberry, Israel	42*	5	28
Str37	N.D.	N.D.	Strawberry, Israel	14	10	45*
Str39	N.D.	N.D.	Strawberry, Israel	23	18	40*
Str41	N.D.	N.D.	Strawberry, Israel	9	48*	55*
Str48	N.D.	N.D.	Strawberry, Israel	18	40*	0
Str49	N.D.	N.D.	Strawberry, Israel	10	40*	3
Str53	N.D.	N.D.	Strawberry, Israel	40*	38*	3
Str54	N.D.	N.D.	Strawberry, Israel	7	18	10
Str59	N.D.	N.D.	Strawberry, Israel	14	53*	33*
Str65	N.D.	N.D.	Strawberry, Israel	36*	10	25
Str76	N.D.	N.D.	Strawberry, Israel	22	8	25
Str79	N.D.	N.D.	Strawberry, Israel	40*	8	10
Str80	N.D.	N.D.	Strawberry, Israel	43*	3	33*
Str82	N.D.	N.D.	Strawberry, Israel	18	3	38*
Str83	N.D.	N.D.	Strawberry, Israel	36*	10	13
Str84	N.D.	N.D.	Strawberry, Israel	48*	10	10
Str85	N.D.	N.D.	Strawberry, Israel	55*	3	5
Str86	N.D.	N.D.	Strawberry, Israel	0	3	25
Str87	N.D.	N.D.	Strawberry, Israel	22	5	18
Str88	N.D.	N.D.	Strawberry, Israel	23	0	33
Str89	N.D.	N.D.	Strawberry, Israel	36*	8	45*
Str92	N.D.	N.D.	Strawberry, Israel	28	5	43*
Str98	N.D.	N.D.	Strawberry, Israel	44*	5	20

Table 1 continued

Isolate #	Accession	Anastomosis groups	Isolate origin	Virulence, %[a]		
				Fruit	Seedlings	
				25°C	25°C	30°C
Str100	N.D.	N.D.	Strawberry, Israel	0	3	10
Str108	N.D.	N.D.	Strawberry, Israel	55*	0	5
Str117	N.D.	N.D.	Strawberry, Israel	18	13	40*
Str118	N.D.	N.D.	Strawberry, Israel	3	5	5
FSR-052[i]	AY684917	Outgroup	Lily[b12]	N	N	N

[a] Data of % necrosis (on fruit) or % mortality (on seedlings) marked with an asterisk are significantly higher than those of the corresponding non-inoculated control ($\alpha = 0.05$ one way ANOVA and post-hoc Dunnett)

[b] ITS sequence obtained from: [b1] Present Study; [b2] GenBank—Kuramae et al. (2003); [b3] GenBank—Kuninaga et al. (1997); [b4] Kuninaga, S. [b5] GenBank—Boysen, Borja, del Moral, Salazar, and Rubio (1996); [b6] GenBank—Gonzalez et al. (2001); [b7] GenBank—Toda et al. (2004); [b8] GenBank—Johanson, Turner, McKay, and Brown (1998); [b9] GenBank—Gronberg et al. (2003); [b10] GenBank—Salazar et al. (unpublished); [b11] GenBank—Gonzalez et al. (2002); [b12] GenBank—Yang and Chen (2004)

[c] Str = isolated from strawberry

[d] N = Not tested for virulence

[e] N.D. = Not determined—classification to AGs of isolates from strawberry in Israel

[f] The original SIR-2 belonged to AG-B(o), but was probably replaced with an unknown AG-A isolate and distributed among *Rhizoctonia* researchers and still appears as an AG-B(o) isolate in GenBank

[g] The sequence for isolate BN-37(gb) appears in GenBank as belonging to AG-R. It is possibly of an unknown AG-F isolate, according to its location in the tree (Fig. 1) and according to its % similarity to AG-F isolates. The sequence of the Bn-37 (AG-R) isolate was confirmed in the present work

[h] The original AV-2 isolate is the representative isolate of AG-I. There are two accession numbers with the accurate sequence in GenBank. However, the sequence of AV-2r AJ242898 (Gonzalez et al., (2002) is of another isolate, probably close to AG-L (Hyakumachi et al., 2005)

[i] Isolate of *Athelia* (*Sclerotium*) *rolfsii*, was used as an outgroup for Fig. 1

The reaction mixture was prepared as described by Freeman, Minz, Jurkevitch, Maymon, and Shabi (2000), the PCR programme was as follows: 5 min denaturation at 95°C, 40 cycles of 30 s at 95°C, 30°s at 50°C, 90 s at 72°C and was terminated with 10 min at 72°C. PCR-amplified rDNA products (ca. 0.67 Kb), were extracted from agarose gel using DNA isolation kit (Biological Industries, Beit Ha'emek, Israel). Ligation and cloning into competent cells of *E. coli* DS5 was done with pGEM®-T Easy Vector System kit (Promega, Madison, WI, USA). The pGEM®-T Easy Vector was extracted with Wizard ® Plus Sv minipreps DNA purification system (Promega, Madison, WI, USA) and the Big Dye Terminator DNA sequencing kit (Perkin-Elmer Inc., Branchburg, NJ) was used for determining sequence of the amplicon (ITS 1 and 2 regions including the 5.8S rDNA) using the vector primers SP6 and T7 (White et al., 1990). The sequences of both strands of the DNA were determined using an ABI prism 377 DNA sequencer (Applied Biosystem Inc., Foster City, CA) at Tel-Aviv University.

Percent sequence similarity and cluster analyses of rDNA-ITS region sequences of *Rhizoctonia* spp. isolates

Sequences of each isolate, including the ones obtained from GenBank, were processed and refined using Chromas, version 1.41 (from the worldwide web at: http://www.trishul.gu.edu.au/nconor/chromas) and processed sequences were submitted to 'DDBJ' (DNA Data Bank of Japan, Research Organization of Information and Systems, National Institute of Genetics, Shizuoka, Japan. http://www.ddbj.nig.ac.jp) for multiple alignment. The results were analyzed by Genedoc version 2.5.000 (Nicholas, Nicholas, & Deerfield, 1997) and processed to remove the excess edges according to the smallest sequence size of the isolates in GenBank (total length of 777 bases).

Manual alignment was performed when necessary. Cluster analysis was performed using the neighbour joining (NJ) method in DDBJ and viewed by TreeView version 1.6.6 (Page, 1996). Maximum Parsimony (PAUP) and Maximum Likelihood (MLM) cluster analyses were also generated for the sequences of the isolates using the Phylip Package 3.6 (Felsenstein, 1989) to support the AGs representing clusters of the NJ analysis. Bootstrap analysis using 100 re-samples of the sequence data was carried out. The percent sequence similarity of the ITS region (including the ITS regions 1 and 2 and 5.8S rDNA) was calculated from the data using the MatGat programme (Campanella, Bitincka, & Smalley, 2003).

Virulence tests for *Rhizoctonia* spp. isolates on stolon-propagated strawberry daughter plants (cv. 'Malakh')

Disease-free daughter plants were obtained from a strawberry production nursery (Rahan Meristem LTD, Rosh Hanikra, Israel), where they were propagated from meristem-cultured nuclear mother plants. Before transplanting to soilless mix (a 3:1 mixture of coconut bark and styrofoam, Pele'mix Industries, Katif, Israel) for the pathogenicity test, the mature leaves were removed, roots were trimmed to 6 cm length and the weight of each plant was recorded. Each pot (12.5 cm diam., 9 cm height) containing 700 ml of soilless mix was planted with one strawberry plant (15 pots/treatment). Wheat grains (30/pot) colonized with the tested *Rhizoctonia* spp. isolate were added into the soilless mix around the plants, 24 h after replanting. Plants were grown in the greenhouse at 28 ± 2°C for 5 weeks and fertilized routinely with 1% NPK solution (10:10:10, 100 ml/pot) added after planting and every other week.

At the end of the experiment, plants were harvested, mortality recorded and the surviving plants were divided into roots and crowns with foliage. Fresh and dry weights (obtained by drying at 80°C for 30 h) were also recorded. In this method virulence was based on weight loss rather than on disease symptoms (Botha et al., 2003; Martin, 1988, 2000). Weight loss was calculated by the differences in weight of each of the inoculated plants from that of the non-inoculated control plants, and by the difference of the gained weight (plant weight of each plant at the end of the growth minus its weight before replanting) of the inoculated plants from that in the non-inoculated control plants. Comparisons were done according to the plant weight gained during the experimental duration (by subtraction of the plant weight of each plant before replanting from that at harvest time, while the initial dry weight was calculated by extrapolation according to an average percentage of dry weight of plants sampled before replanting). The experiments were performed in a complete randomized design and after several preliminary experiments they were repeated twice.

Virulence tests for *Rhizoctonia* spp. isolates on detached strawberry fruit (cv. 'Malakh')

Fruits were collected from organically grown field plants (Tzofit, Sharon region, Israel). Four developmental stages of strawberry fruits were used in a preliminary experiment: a. 7 mm (green); b. 18 mm (green); c. 25 mm (green); and d. 35 mm (red). After removing the calyx, the fruits were surface-disinfested with 1% NaOCl followed by a 40% ethanol solution, washed thoroughly with sterile chloramphenicol (250 μg ml^{-1}) solution, and placed on a moist paper towel covering a metal grid in sterile plastic boxes (6 fruits/box, 2 boxes/treatment—12 fruits/treatment). A wheat grain colonized with the tested isolate was placed next to each fruit and the boxes were covered with aerated lids. Fruit necrosis area was evaluated after 6 days incubation at 25°C. Quantification of fruit necrosis was determined by estimation of % necrotic area of the fruit surface. In a number of preliminary experiments, hard rot caused by *Rhizoctonia* spp. developed on the fruits of the first and second stages, but not on the third and fourth stages of fruit development. Fruits of the second stage were found to be most suitable for infection trials. The major fruit inoculation experiments with *Rhizoctonia* isolates were repeated twice in a complete randomized design.

Virulence tests for *Rhizoctonia* spp. isolates on strawberry seedlings (cv. 'Malakh')

Seeds were collected from air-dried, fruit-skins peeled from organically grown strawberry fruits (Tzofit, Sharon region, Israel), collected during December to April and stored dry at 4°C. The seeds were surface-disinfested with 3% NaOCl for 1 h, immersed in H_2O overnight and evenly spread on the surface of sterilized soilless mix in well drained pots (20 × 10 × 10 cm). The soilless mix was previously watered with 100 ml l^{-1} of 1% KNO_3 solution and covered with a thin layer of vermiculite for germination. The pots were covered with plastic sheets and placed under continuous fluorescent light. In the preliminary test, seedlings with 1, 2 or 3 true leaves were transplanted to pots (10/pot with 250 ml of soilless mix, 4 pots/treatment—40 plants/treatment). Pots were incubated under a 12 h photoperiod (fluorescent light) at 25°C. One wheat grain colonized with the tested *Rhizoctonia* isolate was placed in the soil next to each seedling one day after replanting. According to the conclusions of several preliminary trials, no additional seedling mortality occurred 15 days post-inoculation. Therefore, seedling mortality was recorded 20 days after inoculation. As one-true-leaf plants survived poorly after transplanting and the three-true-leaf plants were less infected by the virulent *Rhizoctonia* isolate compared to the two-true-leaf plants, the majority of the experiments for virulence of the isolates were carried out with two-true-leaf seedlings at 25°C and at 30°C and were repeated twice in a complete randomized design.

Analysis of data

Results were analyzed by STATISTICA version 7.1. (data analysis software system StatSoft, Inc. 2005). Percent seedling mortality or fruit necrosis values were transformed by arcsin ($X^{1/2}$) transformation and analyzed according to one-way ANOVA, followed by post-hoc Dunnett test (>non-inoculated control in each experiment) at a probability level of $\alpha = 0.05$. Results of weight differences (plantlets) were analyzed before transformation to percentage of the control, according to one-way ANOVA, followed by post-hoc Dunnett test (<non-inoculated control in each experiment) at a probability level of $\alpha = 0.05$. All of the data presented in this study are of combined analyses from repeated experiments that indicate no appreciable difference between experiments.

Results

Clustering of *Rhizoctonia* spp. isolates based on rDNA-ITS sequence analysis compared with anastomosis grouping

Seventy-five *Rhizoctonia* spp. isolates obtained from wilted strawberry plants of various cultivars (Str# isolates) collected from fields and nurseries throughout Israel, were tested for their virulence on strawberry (Table 1). Since multiple isolates with the same morphology could be obtained from each location, only a subset of 43 isolates (60%) was selected for their identification to specific anastomosis groups (AGs) representing differences and collection sites. Of these, 23 isolates belonged to BNR AG-A; 10 to AG-F, 6 to AG-G and 1 to AG-K. None of the tested BNR Str isolates from Israel belonged to AG-I. The three multinucleate *Rhizoctonia* (MNR) isolates belonged to AG 4 subgroup HG-I. In addition, one soil isolate from Israel belonged to AG 4 HG-III. Cluster analysis was performed with the rDNA-ITS sequences of Str isolates, representatives of known AGs and of isolate sequences retrieved from GenBank. The sequences clustered consistently in the NJ tree according to the AGs and subgroups. (Fig. 1). The sequences of the isolates in the clusters of this tree were supported by the Maximum Parsimony (PAUP) and Maximum Likelihood (MLM) cluster analyses.

The pairwise percent similarities of the rDNA ITS sequences (according to MatGat) of isolates belonging to the same AG and subgroups were calculated to evaluate the extent of the similarities and differences among the isolates within and among the AGs and subgroup clusters (Table 2). The similarity range within the AGs or subgroups was from 93% to 100%, although

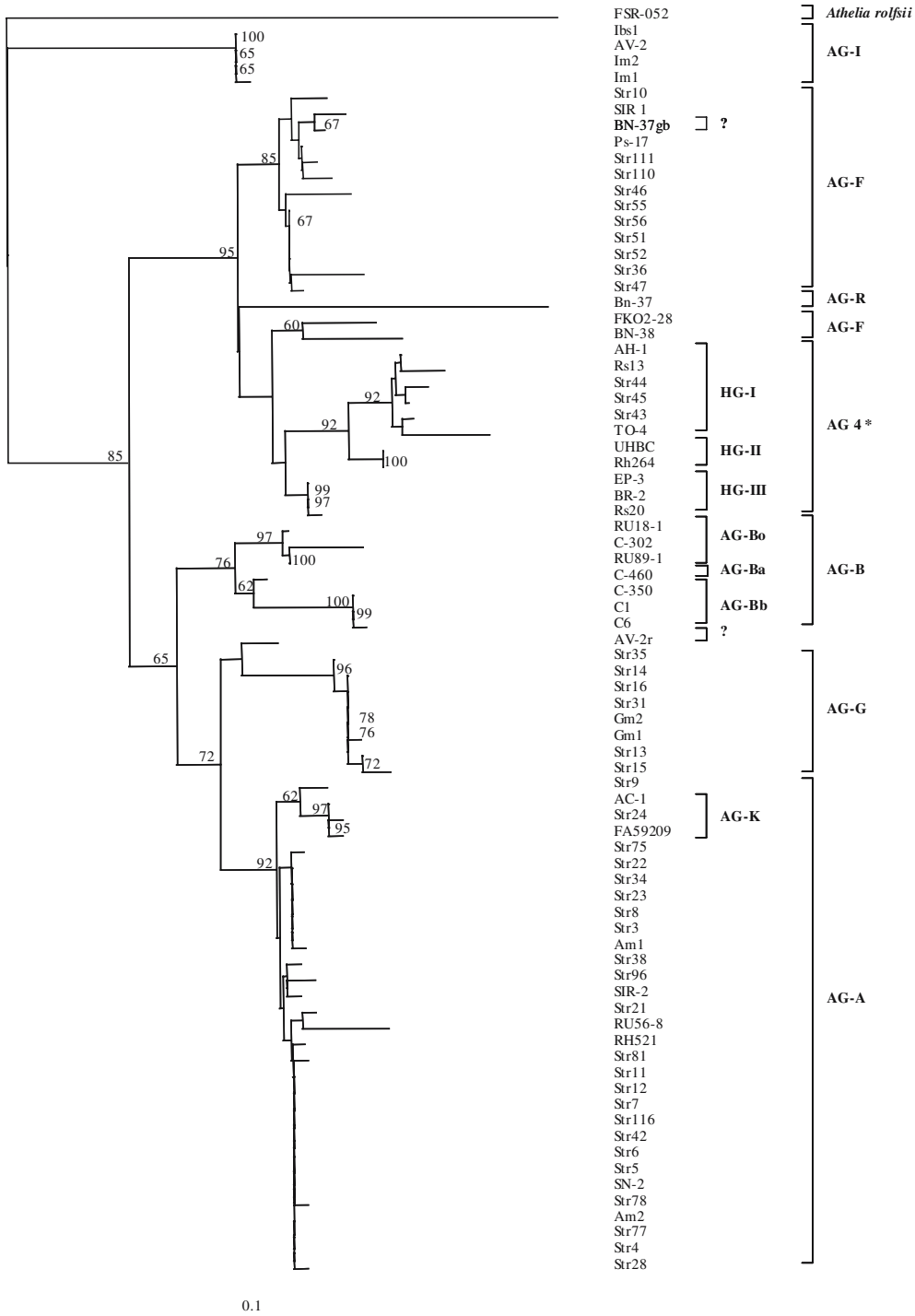

Fig. 1 A neighbour joining tree of *Rhizoctonia* spp. isolates from strawberry, culture collections and GenBank, clustered according to multiple alignment of rDNA-ITS sequences. The distances were determined according to Kimura's two-parameter model and the bar indicates a one-base change per 10 nucleotide positions. Bootstrap values over 60 (of 100 trials) are positioned alongside the branches. Isolate FSR-052, *Athelia* (*Sclerotium*) *rolfsii*, was used as an outgroup; (its horizontal line in Fig. 1 represents only 25% of its actual distance). The AGs and sub-groups for the clusters are indicated. The tree number in the TreeBase is: SN2407. * Except for AG 4, which is MNR, the rest are BNR AGs

Table 2 Summary of ranges in % sequence similarity of rDNA-ITS sequences of *Rhizoctonia* spp. isolates within and between anastomosis groups and subgroups

AG		A	K	G		B			4			F(II)		R	F(I)		I
	Subgroup/isolate				AV-2r	Bb	Ba	Bo	HG-I	HG-II	HG-III	FKO2-28	BN-38			BN37gb	
A		96–100															
K		93–95	99														
G		89–92	89–90	98–100													
	AV-2r	91–92	90	96–97	a												
B	**Bb**	79–82	81–82	80–81	82	99–100											
	Ba	85–87	87	85–86	87	88	a										
	Bo	85–89	86–87	87–88	88	85–86	92–93	99–100									
4	HG-I	79–82	81–83	82–83	83–84	79–81	81–82	81–82	97–100								
	HG-II	80–82	82	81–82	83	81	82	81–82	95–97	100							
	HG-III	79–82	81–82	81–82	82	82	81	80–81	90–92	91–92	100						
F(II)	FKO2-28	83–86	86	86	87	81	85	85	88–89	88	87	a					
	BN-38	84–86	85–86	86	86	81	86	85	88–89	89	87	95	a				
R		79–81	82	81–82	82	80	82	80	88–90	89	88	88	87	a			
F(I)		82–87	82–86	84–88	85–88	80–81	83–86	81–85	88–91	89–90	86–89	89–93	89–92	87–90	93–100		
	BN37gb	85–87	86	87–88	88	80	86	84–85	87–90	89	88	92	92	88	94–99	a	
I		84–87	85	86–88	87–88	80–81	84–85	85	79–80	79–80	80	83–84	82	80	81–85	83–84	99–100
Athelia rolfsii		63–66	64–66	64	65	65	64	64	65–67	66	64	63	66	64	65–66	65	65

[a] Because there is a sequence of only one isolate, a range could not be calculated

for most AGs or subgroups the range was narrower (96–100%), while among the AGs or subgroups it ranged only from 89% to 95%. These results indicate that a % sequence similarity threshold differentiating among AGs or subgroups could not be definitely determined, despite the fact that isolates of different AGs and sub-groups were located in distinct clusters and sub-clusters (Fig. 1), because there was some congruence in percent sequence similarity ranges within and among AGs. The % sequence similarities of isolates of AGs infecting strawberries (including known sequences from GenBank) (28, 3, 8, 3, 6, and 13 isolates, within AG-A, AG-K, AG-G, AG 4 HG-III, AG 4 HG-I, AG-F and AG-I respectively), ranged in their % similarities within each AG from 96% to 100%, 99%, 98% to 100%, 100%, 99% to 100%, 93% to 100% and from 99% to 100% respectively (Table 2).

The sequences of AG 4 isolates were further sub-clustered into three subgroups (HG-I, -II and -III). While the % sequence similarity within AG 4 isolates as a whole ranged from 90% to 100%, it was considerably higher within the subgroups: 97–100, 100 and 100% for HG-I, -II and -III respectively. The sequences of three Str isolates that grouped in one sub-cluster with Rs13 had 100% sequence similarity compared with isolates belonging to the AG 4 subgroup HG-I (Kuninaga, Natsuaki, Takeuchi, & Yokosawa, 1997; Kuramae, Buzeto, Ciampi, & Suoza, 2003), while Rs20 (a soil isolate from Israel, virulent on strawberry fruit) had 99% sequence similarity with isolates that belong to HG-III (Gonzalez, Carling, Kuninaga, Vilgalys, & Cubeta, 2001; Kuramae et al., 2003). The % sequence similarity range among the subgroups within AG 4 was lower, from 90% to 97%. The AG F isolates were located in two distinct clusters. One included the sequences of 10 BNR Str isolates, while the other included the sequences of two GenBank isolate sequences, BN-38 and FKO2-28. While the % sequence similarity range for the isolates within the first cluster was 93–100% and for the second cluster was 95%, the range between these clusters was only 89–93%.

AG-R is located between the two AG-F clusters, which are located close to AG 4 in the tree. Furthermore, the % sequence similarities among AG-F, AG-R, AG 4 HG-I and HG-II (87–91%), were also relatively higher than with the other AGs (79–88%). This may indicate a certain closer genetic relatedness between these BNR and MNR AGs. Despite this relatively closer genetic relatedness, none of the AG-F or AG-R isolates anastomosed with those from AG 4.

The sequence for isolate BN-37(gb) was identified in the GenBank as an AG-R (syn. CAG-5) representative, while its sequence was clearly located within one of the AG-F clusters. However, the sequence of the isolate BN-37 (confirmed in the present work), which is an AG-R isolate (according to anastomosis) is located in a separate location on the tree for AG-R (Fig. 1). This indicates that an inaccurate sequence (quite possibly of an unknown AG-F isolate, according to its location in the tree, Fig. 1) was deposited in the GenBank for this isolate.

The non-pathogenic isolates RU18-1 and RU89-1 anastomosed at high frequencies with the AG-B(o) representative isolate (C-302) and at a lower frequency (<30%) with the representative isolates of AG-Ba and AG-Bb (data not shown). Both isolates have a high sequence similarity (99%) with isolate C-302 and are located in a separate sub-cluster for AG-B(o) (Sneh et al. 1991) and it has been reported as such in several publications (Cubeta, Echandi, & Vilgalys, 1991; Gonzalez et al., 2001; Kasiamdary, Smith, Scott, & Smith, 2002; Otero, Ackerman, & Bayman, 2002). However, its rDNA-ITS sequence deposited in GenBank (and used in the above cited studies) is clearly located within the AG-A cluster. According to anastomosis reaction and location in the tree cluster, the protective non-pathogenic isolate Rh521 and RU56-8 (Sneh & Ichielevich-Auster, 1998) clearly belong to AG-A.

Virulence tests for *Rhizoctonia* spp. isolates on stolon-derived strawberry daughter plants

The most significant reductions of strawberry plant weight inoculated with *Rhizoctonia* isolates were obtained when the comparisons were calculated according to the plant dry weight gained during the experimental duration. Plants inoculated with the various isolates generally gained

less weight (from 21% to 58%) than the non-inoculated control plants (Fig. 2). Four of the isolates (Rs13, Gm1, Am1 and the non-pathogenic RU89-1) caused a significant weight loss compared to the non-inoculated control, while the weight loss caused by isolates Str45, Str28 and Str14 was not significant, although these isolates were significantly virulent by the other test methods (Fig. 2). Isolate Str14 caused a slight increase in plant weight compared to the control plants.

Virulence tests for *Rhizoctonia* spp. isolates on detached strawberry fruit

There was a continuum of virulence (expressed as percent necrosis rates of fruit hard rot) caused by the various isolates belonging to AG 4 (HG-I and HG-III), AG-A, AG-G, and AG-F (Table 1 and Fig. 3). The known pathogenic isolates Rs13, Rs20 and Am1 developed severe necrotic symptoms (100%, 40% and 91%, respectively) compared with the non-inoculated control and fruits inoculated with the non-pathogenic isolate RU18-1 (0%). Fruit inoculated with the known virulent isolates Gm1 (AG-G) or Im1 (AG-I) developed reduced hard rot necrotic areas (35% and 25%, respectively) than fruits inoculated with the known virulent isolates Rs13 (AG 4) or Am1 (AG-A), as well as fruits inoculated with the non-pathogenic isolate RU89-1 [AG-B(o)] (30%). Latent fruit infections with *Botrytis cinerea* (which is scarce in the first month of the fruit-bearing season and is more apparent at the latter part of the season) occurred on some of the fruits. Nevertheless, hard rot symptoms caused by *Rhizoctonia* could frequently be well distinguished from the soft rot and grey mold caused by *Botrytis*, while the fruits which were infected with *Botrytis* were discarded from the data.

Virulence tests for *Rhizoctonia* spp. isolates on strawberry seedlings

In several preliminary experiments, strawberry plants at the two-true-leaf stage were found to be the most suitable for infection with *Rhizoctonia* spp. isolates. While only 70% of the one-true-leaf seedlings survived the transplanting (without inoculation with the pathogen), 97.5% of the two-true-leaf seedlings survived that procedure. Seedling mortality progressed from the third to the 15th day after inoculation, while afterwards (up to 10 weeks), no further significant mortality occurred. Two-true-leaf seedlings were susceptible to virulent *Rhizoctonia* isolates, and when grown at 25°C, isolates RS13, Am1, Gm1, and Im1 caused 75%, 82%, 55% and 30% mortality, respectively. Although three-true-leaf seedlings survived the transplanting, they were less susceptible to the virulent isolates (RS13 and Gm1 caused only 10% mortality). Based on these

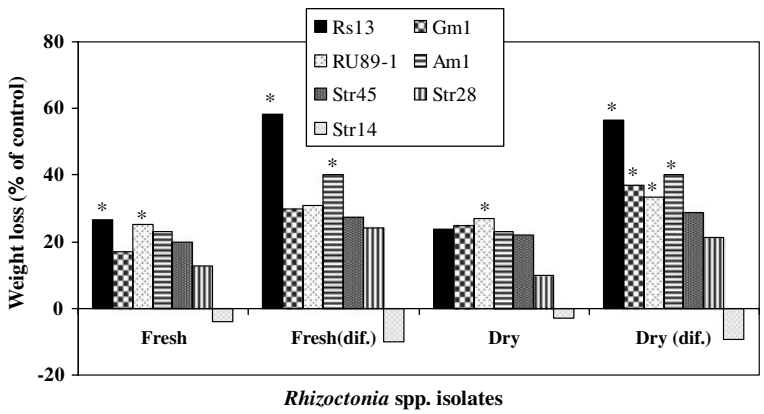

Fig. 2 Percentage of weight-loss of strawberry daughter plants caused by *Rhizoctonia* spp. isolates (the controls were affix to zero and omitted from the figure), 5 weeks after transplanting (dif. = difference). Treatments significantly different from the control, within the same analysis, are marked with an asterisk ($\alpha = 0.05$ one way ANOVA followed by Dunnett test)

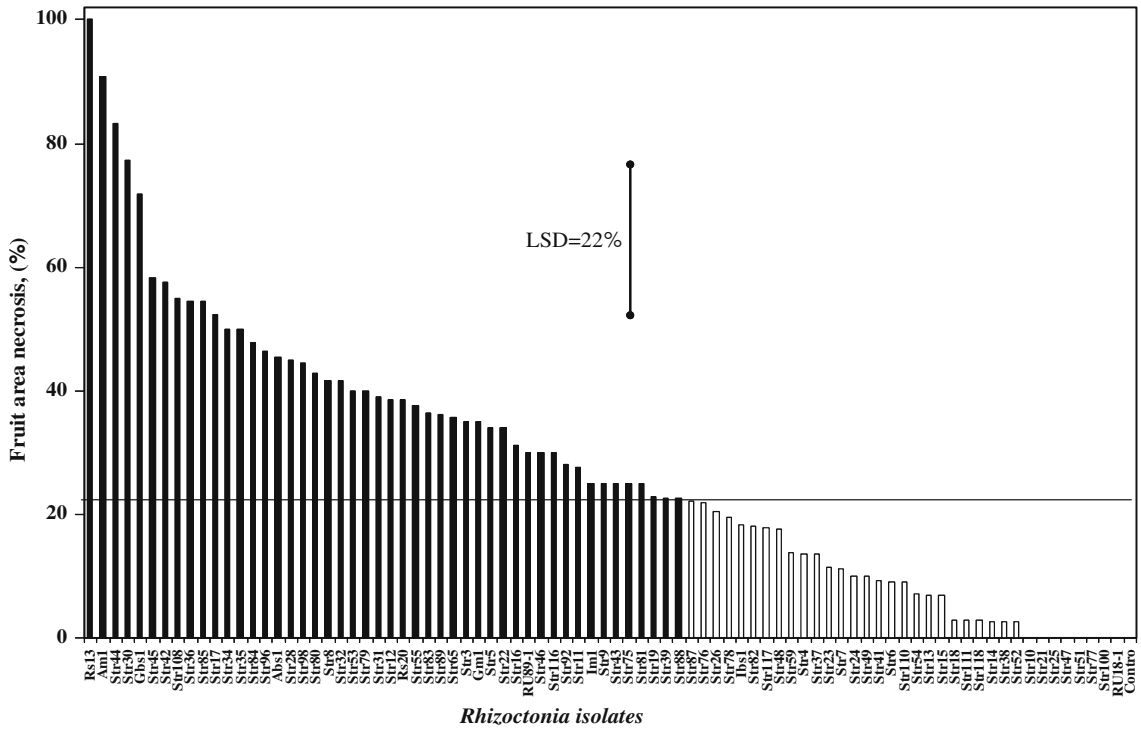

Fig. 3 Percentage of strawberry fruit-area necrosis caused by *Rhizoctonia* spp. isolates after 11 days incubation at 25°C. The horizontal line indicates significant difference threshold from the control (one way ANOVA)

results of the preliminary trials, two-true-leaf seedlings were selected for use in the subsequent virulence tests.

In addition to the known virulent and non-pathogenic *Rhizoctonia* spp. isolates, 75 Str isolates were tested for their virulence on two-true-leaf seedlings at both 25°C and 30°C. Generally, the various isolates caused a continuum in percentage of plant mortality, from 83% (at 25°C) and 75% (at 30°C) to 0% (Table 1). The virulent isolates belonged to AGs -A, -F, -G, -K and AG 4. While some of the isolates were highly virulent at both temperatures, certain isolates were highly virulent at 30°C but did not cause any significant seedling mortality at 25°C compared to the respective non-inoculated control plants. Other isolates were highly virulent at 25°C but did not cause any significant seedling mortality at 30°C. The virulent isolate of AG-I (Im1 from the USA) was weakly virulent at 25°C (caused 30% mortality), but was not virulent at all at 30°C (only 3%).

When virulence of selected virulent isolates [Rs13 (AG 4); Gm1 (AG-G)] and a non-pathogenic isolate [RU89-1 (AG-B(o)] were compared (Fig. 4), the virulent isolates proved to be virulent by all the three methods. The non-pathogenic isolate was categorized as non-pathogenic by two of the methods. Although it belongs to AG-B(o) (isolates of this AG are not known to be pathogenic on strawberry) it caused a significant reduction in plant dry weight when inoculated on daughter plants (Fig. 4).

Some isolates, which were virulent on strawberry fruits, were not virulent on seedlings, while some of the isolates, which were virulent on seedlings, were not virulent on the fruits. For example, isolate Str14 (AG-G) was virulent on seedlings but was not virulent on fruits or on daughter plants (Table 1 and Fig. 2).

Discussion

Most of the BNR *Rhizoctonia* spp. isolates obtained from diseased strawberry plants in Israel, which were classified to AGs belonged to AG-A, while fewer isolates belonged to AG-F

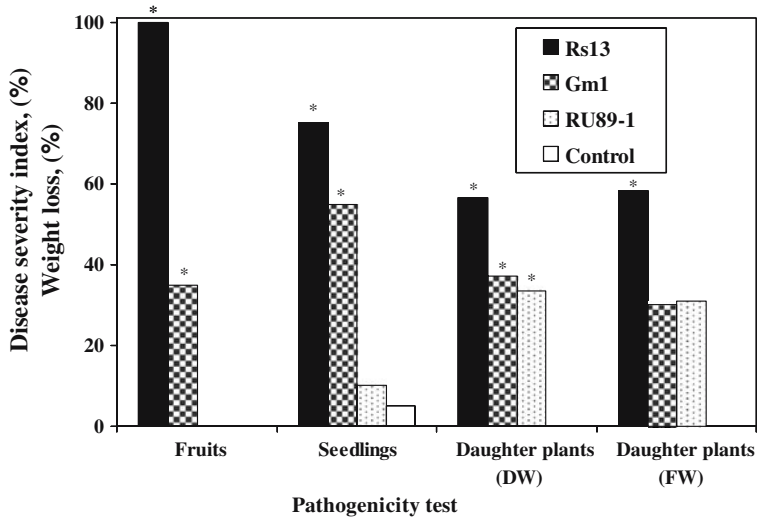

Fig. 4 Disease severity index (DSI) caused by *Rhizoctonia* spp. isolates: A summary of the three different pathogenicity test methods on: (1) young fruit (% fruit necrotic area); (2) seedlings at 25°C (% mortality); and (3) daughter plants weight difference dry- (DW) or fresh-weight (FW) loss, % of the control. Treatments significantly different from the control within an experiment are marked with an asterisk (one way ANOVA followed by Dunnett test, $\alpha = 0.05$)

and AG-G. One isolate belonged to AG-K. Concurrent with the present study, Manici and Bonora (2007) indicated that rDNA-ITS sequences of Italian isolates clustered with AG-K isolates which were virulent to strawberry (citing some of the Str isolates from our present study). None of the BNR isolates from Israel belonged to AG-I. The rDNA ITS sequences of the isolates were located in distinct clusters in the NJ tree, according to their AGs, including AG-I isolates, which were obtained from other sources. The relative virulence of *Rhizoctonia* spp. isolates belonging to various AGs, have been reported to be dependent upon incubation temperatures and nutrition (Martin, 1988). Since AG-I isolates are known to infect strawberry plants at comparatively lower temperatures, and disease symptoms of *Rhizoctonia* spp. appear on strawberry in Israel during the hot summer months, AG-I isolates may be less relevant as strawberry pathogens in Israel. In the present study, a representative AG-I isolate (from California) was mildly virulent on inoculated fruit and seedlings at 25°C, but was avirulent at 30°C. In Connecticut, AG-I isolates were recovered at the highest frequency (72%) in the autumn, while in the spring, fewer isolates (15–26%) belonged to this group. Isolates of AG-G were recovered at the highest frequency (43–56%) followed by AG-A (14–26%) in this season (Martin, 1988). In California, most of the isolates belonged to AG-A (68%), fewer to AG-I (21%) and the fewest to AG-G (11%) (Martin, 2000). In South Africa, most of the BNR isolates belonged to AG-A (69%) fewer to AG-G (25%) and even fewer to AG-I (6%) (Botha et al., 2003). Isolates of AG-F and AG-K, which have not been previously reported as pathogens of strawberry, were found in the present study for the first time to be virulent on strawberry plants.

Of the MNR isolates from Israel, three isolates (from strawberry plants) belonged to AG 4 subgroup HG-I, while another pathogenic soil isolate belonged to AG 4 HG-III. AG 4 isolates (*R. praticola*, teleomorph—*Thanatephorus praticola*) are known to be virulent on a wide plant host range and commonly occur in field soils in different warm geographic regions (Sneh et al., 1991). In the present study, the AG 4 isolates were virulent to strawberry, while isolates of this AG have not been previously reported to infect this crop. In South Africa, all of the MNR strawberry isolates belonged to AG-6. Botha et al. (2003) emphasized that they were more virulent to strawberry plants than the BNR (of

AG-A, AG-G and AG-I). Previously, AG-6 isolates were considered to be weakly virulent on several plant hosts (Sneh et al., 1991). Among the *Rhizoctonia* spp. isolates obtained from strawberry plants in California, only one (0.8%) was multinucleate (Martin, 2000), and similarly, in Connecticut 1–3% of the isolates belonged to AG 5 (Martin, 1988). Members of AG 5 have previously been considered to be only weakly pathogenic on different crop plants (Sneh et al., 1991) and such was the case on strawberry (Martin, 1988). However, isolates of this AG were highly virulent to apple roots and significantly involved in the apple replant problem in Washington State (Mazzola, 1997).

A significant number of the Str isolates were of low virulence, or did not cause disease symptoms despite the fact that they had been isolated from strawberry plants with typical black rot symptoms. It would be interesting to study their role on strawberry plants in the field, considering the potential of hypovirulent *Rhizoctonia* spp. isolates in protection of plants against virulent *Rhizoctonia* spp. isolates (Sneh, 1996) as well as their effect as plant growth promoters (Sneh et al., 1986).

Anastomosis is the most widely used, and popular method for classification of *Rhizoctonia* spp. isolates (Carling, 1996, Sneh et al., 1991). However, it may not be suitable for identification of certain *Rhizoctonia* isolates, such as those that anastomose with representatives of more than one AG (isolates of AG BI, now considered belonging to AG 2, anastomose also with isolates of AGs 2, 3, 6 and 8), indicating that some of the AGs are actually bridging groups (Carling, 1996; Sneh et al., 1991). On the other hand, some isolates have even lost their capability to self-anastomose (Hyakumachi & Ui 1987). For such isolates, the anastomosis grouping method may not prove accurate for classification. In BNR, which have relatively narrow hyphae, it was shown that pairing of certain isolates with tester isolates of the same AG did not always result in a positive anastomosis reaction, even though both isolates did anastomose with other members of the same AG (Martin, 2000). Using molecular methods, clustering in a tree based on rDNA-ITS sequence analysis (and % similarity of the sequences) in combination with anastomosis, may provide a more accurate way for identification and classification of *Rhizoctonia* spp. isolates.

Cluster analyses based on rDNA-ITS sequences of representatives of certain MNR and BNR AGs and subgroups of *Rhizoctonia* spp. isolates have already been reported (Carling et al., 2002; Gonzalez et al., 2001; Hyakumachi, Priyatmojo, Kubota, & Fukui, 2005; Kasiamdari et al., 2002; Kuninaga et al., 1997; Liu & Sinclair, 1992; Salazar et al., 2000; Toda, Mghalu, Pryatomojo, & Hyakumachi, 2004). In the present study, the *Rhizoctonia* spp. isolates from strawberries revealed that the rDNA-ITS sequences were clustered consistently according to their known AGs. None of the sequenced Israeli isolates (virulent on strawberry) in the present study belonged to the HG-II subgroup of AG 4. rDNA-ITS sequences of AG 4 isolates together with sequences of known AG 4 isolates from the GenBank were located in three distinct subclusters, representing the three AG 4 subgroups: HG-I, II, and III, as has also been shown in a parsimonious tree based on phylogenetic analyses of rDNA-ITS sequences by Gonzales et al. (2001).

Isolates of AG-K are genetically related to those of AG-A. In the NJ tree (Fig. 1) the AG-K cluster was located within the AG-A cluster, which may interfere with separating it from AG-A. The % rDNA-ITS sequence similarity between these two AGs is 93–95%. Compared to the range of subgroups (AG 4 = 90–97%; or AG-B = 85–93%), AG-K seems to be in the same range of a subgroup to AG-A. Nevertheless, the % sequence similarity within these AGs (A = 96–100%, K = 98–100%) and the lack of anastomosis among isolates of these AGs, indicate that they are distinctly separated, though genetically related.

The sequences of the AG-F isolates were located in two clusters. One cluster included sequences of 10 BNR Str isolates and of two isolates from GenBank, while sequences of the two AG-F isolates from GenBank were located in a separate cluster. In addition, anastomosis of isolates between the two clusters exhibited only <30% frequency (in the present study). Percent sequence similarity range between isolates from

these two clusters was relatively low. More work using a significant numbers of AG-F isolates is needed to support the existence of more than one subgroup within AG-F.

The locations of the BNR AG-F clusters are close to that of the MNR AG 4 cluster. Furthermore, sequence similarity between the AG-F isolates and the AG 4 isolates ranged from 86% to 91%, while that between AG-F and AGs -A, -B, -G, -I and -K were relatively lower, and ranged from 79% to 83%, indicating a relatively closer genetic relatedness of ITS sequences between the BNR AG-F to the MNR AG 4 than between BNR AG-F to the other BNR groups. Similarly, AG-R is also relatively close to AG-F and to AG 4.

Using GenBank rDNA-ITS sequences for analyses of only one or two isolates may sometimes be misleading and such errors have already been repeated widely in subsequent publications, leading to incorrect conclusions. The following examples have been demonstrated in the present study. The rDNA-ITS sequence of isolate BN-37(gb) was deposited in the GenBank (Gonzalez et al., 2002) as a representative of AG-R (syn. CAG-5), while its sequence clearly located this isolate within the AG-F cluster. However, the accurate ITS sequence of the original isolate BN-37 (AG-R, performed in the present study), is located in a separate location on the tree from AG-R.

The original isolate SIR2 was a representative isolate for AG-B(o) (Sneh et al., 1991) and it has been reported as such in several subsequent publications (Cubeta et al., 1991; Gonzalez et al., 2001; Kasiamdary et al., 2002; Otero et al., 2002; Salazar et al., 2000). However, its rDNA ITS sequence deposited in GenBank (and used in the above cited studies) is clearly located within the AG-A cluster (Fig. 1). Therefore, the representative isolate that had been distributed among researchers as SIR2 was undoubtedly replaced by mistake with an unknown AG-A isolate (confirmed by Ogoshi, personal communication, whose culture collection served as the source for the representative isolates). According to anastomosis reaction and location in the tree cluster, the protective non-pathogenic isolates Rh521 and RU56-8 (Sneh & Ichielevich-Auster, 1998) clearly belong to AG-A. In the phylogenetic tree of Gonzalez et al. (2001) this isolate was also located in the same cluster as an AG-A isolate, but there was no description for its unclear location on this tree. Isolate CFM1 (Kasiamdary et al., 2002) is similarly inaccurately designated as belonging to AG-B(o). In the present study, isolate C-302, a correct representative isolate for AG-B(o), was used as an AG-B(o) representative and the two non-pathogenic isolates RU89-1 and RU18-1, which anastomosed with this isolate and had a high sequence similarity (98%) to this isolate and were closely spaced sub-clusters in the tree, belong to the same AG. Isolate RU89-1 was already previously identified as an AG-B(o) isolate (Sneh & Ichielevich-Auster, 1998). The three AG-B subgroups were located on close sub-clusters in the tree.

Isolate AV-2 is the representative isolate for AG-I (Sneh et al., 1991), and its sequence is also located with the other AG-I isolates in the same cluster in the present study. In the GenBank there are three accession numbers for the AV-2 isolate. The accession: AJ419932 (Gronberg, Paulin, & Sen, 2003) and AB196650 (Hyakumachi et al., 2005) are identical and of the correct AV-2 isolate. However, the third sequence, AJ242898, considered as AV-2 (AV-2r from the lab of V. Rubio) (Gonzalez et al., 2002), is distinctly different and is undoubtedly of another unknown isolate. This sequence was also used in another study inaccurately as an AG-I isolate but it clustered with two AG-L isolates (Hyakumachi et al., 2005). Additional work is required to determine the correct AG of this isolate. The non-pathogenic isolate Rh521 was inaccurately identified previously as an MNR AG 4 isolate (Ichielevich-Auster et al., 1985; Sneh & Ichielevich-Auster, 1998) and, subsequently, as a BNR isolate (Gonzalez et al., 2001, 2002; Salazar et al., 2000). Isolate RU56-8 was inaccurately identified as belonging to AG-P (Sneh & Ichielevich-Auster, 1998). In the present study both isolates were identified to AG-A by both anastomosis and sequence clustering.

The present study indicates that a relatively wide rage of percent similarity was found for sequences within the BNR AGs studied. Therefore, statistically, the probability of obtaining more reliable representative results is expected when more isolates of the same group are

analyzed. A threshold of percent similarity of ITS sequences for differentiating isolates belonging to different AGs or subgroups could not be determined, because in some instances there was an overlap between the percent sequence similarity range for isolates within an AG with the % similarity range between the different AGs. Cluster locations of different AGs in trees based on ITS sequence analysis are calculated by the relevant computer programme used, yet there are additional differences among the isolates, which are not expressed only by the % sequence similarity.

Few studies have examined the genetic diversity of the ITS sequences of isolates within and among AGs, and it has been reported that although few isolates of BNR were investigated, their genetic variations were higher than those of MNR isolates (Gonzalez et al., 2001). In the present study, the percent rDNA-ITS sequence similarities were calculated in a pair-wise manner and ranged between 93% and 100% similarities for isolates within the various AGs or subgroups and 79–95% for isolates among the different limited number of AGs and subgroups.

The currently practiced method for determining virulence of *Rhizoctonia* spp. isolates is based on inoculation of stolon-derived daughter plants and evaluated by the extent of reduction in plant biomass, rather than on typical disease severity symptoms (Botha et al., 2003; Liu & Sinclair, 1992; Martin, 1988, 2000; Wilhelm et al., 1972). Such methods are slow, laborious and require large amount of space. They are also limited to the season of daughter plant availability and standardization of the stolon daughter plant size, age and developmental stage of the root system. A known non-pathogenic isolate (RU89-1), which belongs to AG-B(o) (also a group which is not known to be pathogenic on strawberry), was non-virulent on seedlings and fruit, but significantly reduced the plant biomass of daughter plants, indicating that according to this currently practiced method it may be considered virulent. Furthermore, isolate Str14 that was highly virulent on seedlings did not reduce, and even caused a slight increase in plant biomass of daughter plants. These results indicate that this virulence test failed to identify this isolate as virulent. These results indicate that various isolates may differ in their virulence to different stages of plant growth or to different plant organs. Further work is needed to determine whether certain isolates are actually more specific in their ability to infect only certain organs of the strawberry plant, and what roles these isolates play in inciting disease under different field conditions worldwide. This information is important for evaluating the relevance of the virulence test method for *Rhizoctonia* spp. isolates as they appear in the field and in breeding programmes for resistance. Since virulence of isolates differs at different temperatures, accurate virulence testing may require incubation of the inoculated host plants at a range of temperatures, for example, at 30°C, 25°C and at approximately 15°C to cover cooler temperature isolates such as those of AG I.

The virulence test method based on inoculation of strawberry seedlings may be more reliable among the methods tested for virulence of *Rhizoctonia* spp. isolates. It is based on typical disease symptoms (seedling mortality) is less variable, less time-consuming, occupies less growing space, and harvested, stored seeds can be made readily available throughout the year. This is in contrast to the runner-derived daughter-plant method, which is based on reduction in growth and not on typical disease symptoms.

Acknowledgements We thank Dr. F.N. Martin, USDA-ARS, Salinas, California, for the BNR isolates; Rahan Meristem Nursery for providing the strawberry daughter plants; E. Gamliel-Atinsky, Y. Denisov, S. Horowitz, M. Maymon, G. Prudovsky, A. Zveibil, Y. Agmon and Y. Wolf for their valuable assistance.

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