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FULL RESEARCH PAPER

Molecular genetic variability of Italian binucleate *Rhizoctonia* spp. isolates from strawberry

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Abstract Fifty-eight binucleate *Rhizoctonia* isolates were collected over six years from strawberry plants displaying symptoms of black root rot in Italy. Almost all isolates were able to produce necrosis on strawberry roots, most of them also showed this ability on faba bean and, with lower frequency, on a crucifer and a cereal crop used in rotation with strawberry in Italy. The sequence alignment of Internal Transcribed Spacer (ITS) regions of 51 binucleate *Rhizoctonia* were analyzed and compared with a set of eight sequences representative of *Rhizoctonia* isolate Anastomosis Groups (AG) already found to be pathogenic on strawberry (AG-A, AG-G, AG-I and AG-F). The neighbour-joining tree, based on ITS region sequences, divided Italian strawberry *Rhizoctonia* isolates into two main clusters corresponding to AG-A and AG-G. The results were confirmed by hyphal anastomosis tests. The clustering obtained with the phylogenetic tree was also confirmed using PCR-Restriction Fragment Length Polymorphism of 28S rDNA to compare some isolates, defined as AG-A and AG-G on the basis of ITS region sequence analysis, with representative AG isolates pathogenic on strawberry. The AG-A and AG-G *Rhizoctonia* spp. were widespread in Italian strawberry-growing areas, although with different relative frequencies: AG-G was most frequent in northern (latitude 44°N) and AG-A in southern (latitude 39–40°N) Italy. Analysis of MOlecular VAriance, based on geographic location, showed that *Rhizoctonia* molecular variations between northern and southern Italy accounted for 36.6% of the total, but most of the variations (61%) occurred within each of the four geographical regions from where the isolates originated.

Keywords Black root rot · *Fragaria* × *ananassa* · ITS region · Nucleotide sequence

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Introduction

Rhizoctonia spp. are the main agents of strawberry black root rot, a yield-limiting disease of strawberry (*Fragaria* × *ananassa*) occurring in all the main strawberry-growing areas of the world (Wing, Prittis, & Wilcox, 1994). Although there are many non-lethal pathogens involved in strawberry black root rot, such as *Pythium* spp., *Cylindrocarpon* sp., *Fusarium oxysporum*, *Fusarium* spp. and others (Mena, Palacios de Garcia, & Gonzàles, 1975; Pinkerton, Ivors, Reeser, Bristow, & Windom, 2002; Watanabe, 1977), in most surveys carried out to determine the relative role of various fungal agents involved in black

root rot complex, *Rhizoctonia* spp. are the most frequently isolated fungal pathogens (Manici, Caputo, & Baruzzi, 2005; Pinkerton et al., 2002).

In Italy, strawberry black root rot has always been reported as the main health problem of the crop (Ciccarese & Cirulli, 1983; D'Ercole, 1970; Tamietti & Valmaggia, 1994) and *Rhizoctonia* spp. as the main agent of the root rot complex (Manici et al., 2005; Tamietti & Valmaggia, 1994). In Italy the strawberry crop, with a maximum life span of one year, is cultivated in different climatic areas, from latitude 37° to latitude 44°. The entire area dedicated to this crop (3900 ha in 2003–2004) has decreased over the last 10 years because of high production costs, difficulties in finding labour and the increasing competition on the European market from several Mediterranean countries. The phase-out of methyl bromide and the current restrictions on the use of soil fumigants to control strawberry root rot complex, are additional reasons for crop yield reductions.

Rhizoctonia spp. isolated from strawberry are frequently reported as binucleate *Rhizoctonia*, *R. fragariae*, or *Rhizoctonia* spp., and multinucleate *Rhizoctonia solani* (Elmer & LaMondia, 1999; Molot & Ferière, 1989; Watanabe, 1977). In the United States *Rhizoctonia* from strawberry has been identified in the past as a binucleate or *R. fragariae* (Martin, 1988; Ribeiro & Black, 1971). This is confirmed by Martin (2000) who, using Restriction Fragment Length Polymorphism (RFLP) analysis of a PCR-amplified region of rDNA combined with anastomosis grouping, found AG-A, AG-I and AG-G to be the most frequent anastomosis groups of binucleate *Rhizoctonia* from strawberry.

The rDNA Internal Transcribed Spacer (ITS) region sequence has been broadly used for the identification of *Rhizoctonia*-like isolates (Gonzales et al., 2001) and to study interspecific (Fenile et al., 2003; Kuninaga et al., 1997) and intraspecific (Carling et al., 2002; Hsiang & Dean, 2001) variability of *R. solani* (*Thanatephorus* spp.) groups as regards their hyphal anastomosis pattern. In contrast, very few studies have been done on the variability of *Ceratobasidium*, with which binucleate *Rhizoctonia* has been commonly associated (Vilgalys & Cubeta, 1994), and which seems to be characterized by a greater genetic diversity than that observed within the *Thanatephorus* genus (Gonzales et al., 2001).

A study was carried out on the rDNA ITS region of a sample of binucleate *Rhizoctonia* spp. isolates from strawberry roots with root rot symptoms collected in several Italian environments. The aims of this study were to: (i) sequence the ITS region of the Italian isolates of *Rhizoctonia* spp. from strawberry to identify their relationship with the sequences of *Rhizoctonia* isolates from strawberry already included in GenBank; (ii) evaluate the genetic diversity within Italian isolates and the differences between *Rhizoctonia* agents of black root rot occurring in two main Italian climatic regions, north and south Italy. Finally, as many biotic and abiotic factors are involved in the pathogenic expression of *Rhizoctonia* causing strawberry root rot (LaMondia & Martin, 1989; LaMondia 2003; Molot & Ferière, 1989; Wing et al., 1994) and agronomic tools, such as crop rotation and organic amendments, are reported to increase crop health (Elmer & LaMondia, 1999; LaMondia, 1999), we decided to investigate the level of specialization of strawberry *Rhizoctonia* spp. isolates on the four plant species representing the botanical families most frequently used in crop rotation.

Materials and methods

Fifty-eight *Rhizoctonia* spp. strains were collected in Italy between 1996 and 2001 from strawberry plant roots in the Emilia Romagna region, the main strawberry growing area in northern Italy with a temperate sub-continental climate (north Italy, latitude 44°N), and from three growing areas (Campania, Basilicata and Sardinia) located in south Italy (in latitude 39°N–40°N). *Rhizoctonia* strains were chosen following the same criteria and collected over a six-year survey of black root rot complex in these areas. Feeder roots were collected from each strawberry plant at the end of vegetative growth and at early harvesting stage, washed under running water, disinfected for 2 min in 1% sodium hypochlorite, and rinsed twice with sterile water. Tissue seg-

ments (.3–.5 cm) were excised from the distal, middle, and proximal parts of the two feeder roots of each plant, placed on water agar, and incubated for 3–4 days. All hyphae growing from root segments were transferred to potato sucrose agar (PSA) amended with 200 mg ml^{-1} of streptomycin sulphate. At each sampling time, *Rhizoctonia* like colonies, identified by typical hyphal branching, were grouped on the basis of colony morphology. A few representative isolates from each cluster (varying from 2 to 10 according to the isolate number and morphological variability of isolate sample) were transferred and maintained on PSA in the dark at about 4°C. Representative isolates of *Rhizoctonia* spp. were obtained from a population of more than 800 *Rhizoctonia* isolated from strawberry plant roots collected in ten locations in northern and ten in southern Italy.

Rhizoctonia isolates were identified on the basis of colony morphology and typical hyphal branching pattern. The number of nuclei within the hyphal cells and hyphal fusion were determined using the clean slide technique (Kronald & Stanghellini, 1988). At the beginning nuclei were observed using a light microscope after staining with .5% Aniline blue in lactophenol (Herr, 1970) and with Safranin O, but difficulties encountered in counting intra-hyphal nuclei were completely overcome by using a fluorescent microscope and Acridine orange for staining (Yamamoto & Uchida, 1982). The clean slide technique was also used to test anastomosis among *Rhizoctonia* isolates; the hyphal interactions were examined at 40× and 100× magnification using a light microscope. The anastomosis test was first carried out among isolates belonging to two morphologically defined groups (white-colour colonies and brown-colour colonies). Then the anastomosis test was carried out within the isolates of each group. Finally, the anastomosis reaction test was performed among binucleate *Rhizoctonia* spp. isolates, identified by molecular tools and AG tester AG-A (Str22) and AG-G (Str14), (kindly provided by Prof. Baruch Sneh, Tel Aviv University, Israel).

Pathogenicity

Fifty-eight *Rhizoctonia* spp. isolates were tested for pathogenicity on strawberry (cv. Patty), wheat (*Triticum aestivum*, cv. Centauro; the most frequent crop used in rotation with strawberry), faba bean (*Vicia faba*, cv. Vesuvio; used in rotation in organic systems for nitrogen supply), and on brassica (a selection of *Brassica juncea*, tentatively used as a catch crop in Italy). In all cases, pathogenicity tests were performed using the same experimental design and method for artificial infection as follows: one disk of 6 day-old colonies of each of the 58 *Rhizoctonia* isolates, grown on water agar in 9 cm diam Petri dishes, was placed in each pot (10 cm diam) half filled with a peat–sand mixture (3:1) and then filled with an additional 3 cm layer of peat–sand. Control pots were prepared as above, placing in each pot a sterile water agar disk. Three fresh strawberry plants, with feeder roots no longer than 1 cm, were transplanted into each pot and each pot was considered a replicate. Wheat, faba bean and brassica were seeded in pots artificially inoculated; in this case, 27 seeds for each plant cultivated were arranged in three pots (nine per pot). Plants were grown for 45 days in a greenhouse at 25–30°C under natural light starting from mid-August, then harvested and washed under running water. The presence/absence of root tissue discolouration or necrosis was easy to record at a well-defined root portion of the artificially inoculated plants. A necrosis severity score was assigned to each isolate on the basis of visual observation of three replicates compared to the healthy control, using an exponential scale: 1, healthy plants (non-pathogenic); 3, weak symptoms, tissue discolouration (low pathogenic); 9, necrosis. (pathogenic). To compare the pathogenicity of *Rhizoctonia* spp. isolates on the four tested host plants, Bray-Curtis cluster analysis was carried out to evaluate the pathogenicity using the BioDiversity programme (McAleece, Lambshead, Patterson, & Gage, 1997) on log transformed data.

DNA extraction and sequencing

Each *Rhizoctonia* isolate was grown in potato sucrose broth (PSB) for 3–5 days at 25°C. Fungal mycelium was harvested by filtration on filter paper and stored at –20°C prior to DNA isolation. The frozen mycelium was crushed using a bead

beater (Mixer Mill 300) and tungsten carbide beads. Total genomic DNA extraction was performed with a DNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. PCR amplification of rDNA gene fragments was carried out using primers ITS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990). Amplification reactions (25 µl) were performed using 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 2 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates (dNTPs), .2 µM primers, 2U of Taq polymerase (Invitrogen, Carlsbad, CA) and 1 µl of template. A T-Gradient thermalcycler (Biometra, Goettingen, Germany) was used with the following programme: 1 cycle for 1 min at 94°C; 30 cycles of 15 s at 94°C, 15 s at 58°C, 15 s at 72°C; 1 cycle for 7 min at 72°C.Aliquots of each PCR product were separated by electrophoresis on 1.2% agarose gels stained in ethidium bromide and visualized using a UV transilluminator. PCR products were purified with ExoSAP-IT reaction (Amersham Biosciences, Uppsala, Sweden) and the purified DNA obtained was then directly sequenced at the Bio Molecular Research Service (C.R.I.B.I. Università di Padova) by cycle sequencing using the BigDye sequencing kit with an automated DNA sequencer (Applied Biosystem, Foster City, California) and ITS1 and ITS4 as primers.

Out of the 58 *Rhizoctonia* isolates analyzed in this study, 51 were successfully sequenced. The accession numbers assigned by GenBank to *Rhizoctonia* isolate sequences were AY38627 (R1), AY738628 (R72) and the successive numbers from AY927315 (R2) to AY927363 (R79) to the other 49 *Rhizoctonia* isolates.

Phylogenetic analysis

Sequences obtained from 51 *Rhizoctonia* isolates were checked for complementary strands and the resulting sequences were aligned using the Clustal W software package (Thomson, Higgins, & Gibson, 1994). A phylogenetic tree was inferred from distance matrices, using the neighbour-joining method (Saitou & Nei, 1987), analyzing 51 sequences of binucleate isolates with a set of sequences belonging to eight previously characterized strains representing *Rhizoctonia* binucleate AG types from strawberry retrieved from GenBank (the accession numbers are listed in Table 1). Distances in the ITS rDNA region were determined by Kimura's two-parameter model (Kimura, 1980), omitting all sites with gaps. The topology of the tree was tested by bootstrapping (1,000 re-samplings). Phylogenetic analysis was done using the software package MEGA version 3.0 (Kumar, Tamura, & Nei, 2004)

RFLP analysis

RFLP analysis was carried out to confirm the isolate identification obtained with phylogenetic analysis based on nucleotide sequence. A set of 14 representative isolates (chosen from the main clusters obtained by neighbour-joining analysis) and six strawberry isolates belonging to AG-A (Str22 and Am_1), AG-G (Str14 and Gm_1), AG-I (Im_1) and AG-F (Str36) (kindly provided by Prof. Baruch Sneh, Tel Aviv University, Israel) were compared on the basis of the 28S rDNA RFLP banding patterns. A portion of the 28S rRNA gene from genomic DNA, obtained as already described, was amplified using the primers LR7 and LR0R (Cubeta, Echandi, Abernethy, & Vilgalys, 1991). The PCR reaction was set up in a volume of 50 µl containing 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates (dNTPs), .2 µM primers, 2U of Taq polymerase (Invitrogen, Carlsbad, CA) and 1 µl of template. Amplification was done as follows: 1 cycle for 1 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C; 1 cycle for 7 min at 72°C.

For restriction analysis, aliquots of each PCR product were digested separately with *Taq*I, *Hha*I or *Hpa*II restriction enzymes in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). DNA fragments were separated by electrophoresis on 3% NuSieve GTG agarose gels (FMC Cambrex Corporation, Rockland, ME), stained in ethidium bromide and visualized using a UV transilluminator. Restriction fragment sizes were determined with Low DNA Mass ladders (Invitrogen, Carlsbad, CA) molecular size marker. The experiment was repeated three times.

Table 1 Summary information of the binucleate *Rhizoctonia* spp. used in phylogenetic analysis

Isolate	Anastomosis group	Host	Origin	Accession number
C-662	A	Soil	Japan	AF354092[a]
Str22	A	Strawberry	Israel	DQ102423[b]
Str36	F	Strawberry	Israel	DQ102435[b]
SIR1	F	Sweet potato	Japan	AF354085[a]
Gm1	G	Strawberry	USA	DQ102395[b]
Str14	G	Strawberry	Israel	DQ102402[b]
Ibs	I	Soil	Israel	DQ102442[b]
Im1	I	Strawberry	USA	DQ102443[b]

[a] Gonzales et al. (2001); [b] Sharon et al. (2006)

The sizes and number of initial amplified fragment and digestion products were determined. A data matrix, where 0 was absence of band and 1 presence, was constructed on amplified fragments digested with three different restriction enzymes. A total of 36 character states were included in the analysis. The programme Free Tree (Hampl, Pavlicek, & Flegr, 2001) was used to perform the cluster analysis, using unweighted pair group arithmetic average-linkage algorithm, and the bootstrap analysis, with 1000 repetition count.

Analysis of molecular variability

Analysis of molecular variability (AMOVA) (Excoffier, Smouse, & Quattro, 1992) using the pairwise difference as distance method was carried out on 51 nucleotide sequences aligned with the Clustal W programme. Identifier nucleotide sequences (Schneider, Roessli, & Excoffier, 2000b) were obtained from the ITS1 region of each isolate by visual analysis, and a list of 33 nucleotide-haplotypes were built for distance computation. The data matrix obtained was subjected to AMOVA pairwise *F* statistic and haplotype frequency analysis using ARLEQUIN version 2.0 (http://lgb.unige.ch/arlequin/software/) (Schneider, Roessli, & Excoffier, 2000a).The genetic variability of Italian *Rhizoctonia* isolates was analyzed according to the following hierarchic subdivisions: north and south Italy (first level), while the second level was the geographic regions: Emilia Romagna (north), Basilicata, Campania and Sardinia (south).

Results

Pathogenicity

Forty-eight out of 58 binucleate *Rhizoctonia* isolates were pathogenic on strawberry and 53 infected faba bean, while 35% of the isolates were pathogenic on brassica and only a few (15%) were weakly pathogenic on wheat (Fig. 1). The pathogenicity score assigned for strawberry was always necrosis (pathogenic); despite the variability in severity observed, necrosis symptoms were observed on at least one of the inoculated plants.

Cluster analysis grouped isolates for pathogenicity in three groups; the isolates pathogenic on strawberry and faba bean were in the same cluster, with 78.4% similarity, while isolates were clustered for pathogenicity on brassica and wheat in two separate clusters with respectively 39.3 and 20.5% similarity. The values of similarity (Table 2) showed isolate pathogenicity on four inoculated plants. The correlation coefficient between strawberry and faba bean scores was low, but positive, while the pathogenicity scores of 58 isolates on wheat and brassica were negatively correlated to that recorded on strawberry.

Phylogenetic analysis

Nucleotide sequences were successfully obtained for 51 of the 58 binucleate *Rhizoctonia* isolates considered at the beginning of the study. The neighbour-joining tree, based on ITS region sequence data, revealed that isolates belonging

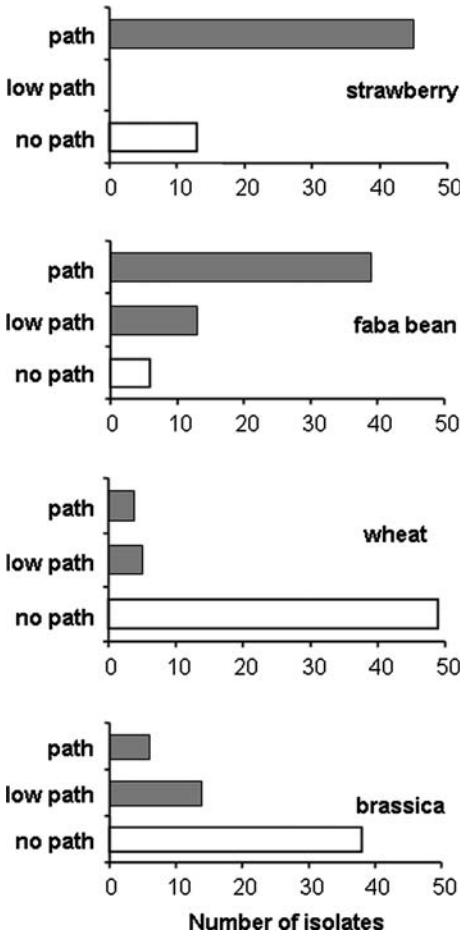

Fig. 1 Pathogenicity (Path) of binucleate *Rhizoctonia* spp. isolates towards strawberry, faba bean, wheat and brassica

Table 2 Similarity matrix obtained with Bray-Curtis cluster analysis of pathogenicity of 58 binucleate *Rhizoctonia* spp. isolates in four tested host plants

	Strawberry	Faba bean	Brassica
Wheat	13.46	13.59	20.51
Strawberry		78.45	39.31
Faba bean			32.75

to Italian strawberry *Rhizoctonia* spp. formed two main groups, with the exception of isolates R7 and R65 (Fig. 2). Sixty-eight percent of the isolates were grouped in a clade with two representative AG-A strains retrieved from GenBank (Fig. 2) with a similarity score varying from 95% to 100%. All AG-A isolates were characterized by a low morphological variability showing a white colony colour without forming sclerotia. AG-A strains were divided by phylogenetic analysis into four sub-clusters, indicating genetic variability. *Rhizoctonia* isolates identified as AG-A on the basis of the result of this analysis showed varying anastomosis ranging from lack of anastomosis to a maximum of 50% with a highly variable frequency. The anastomosis frequency for several of these isolates and AG-A tester isolate was similar, with a higher frequency of imperfect hyphal anastomosis.

The second main group of *Rhizoctonia* isolates, fifteen isolates, clustered together with two representative isolates of AG-G (Fig. 2, Table 1). AG-G isolates showed the highest genetic similarity (from 99% to 100%). They were characterized by a brown colony colour and did not form sclerotia. The inclusion of Italian isolates in AG-G was confirmed by hyphal anastomosis tests with isolates representative of AG-G.

R65 ad R7 were the only sequences outside the main clusters and both were near the main AG-A clade (Fig. 2): R65 had similarity score with AG-A isolates varying from 91% to 98%, while R7 had similarity score with AG-A isolates varying from 90% to 93%. The anastomosis test did not confirm that isolates R7 and R65 belonged to AG-A or to any other available representative AGs, although R65 showed high hyphal affinity (contact) with several isolates of AG-A.

RFLP analysis

PCR amplification of the 28S rRNA portion of *Rhizoctonia* strawberry isolates revealed a variability in number and size of amplified fragment with LR0R and LR7 primers as already seen (Carling et al., 2002; Martin, 2000). The initial amplified fragments had 1.4, 1.6, 1.8 and 3 kb amplified bands. These are reported in Table 3 and they were not included in the data matrix subjected to cluster analysis because they did not change the results obtained with three restriction enzymes.

The cluster analysis represented by a rectangular dendrogram (Fig. 3) grouped *Rhizoctonia* spp. isolates according to the clustering already obtained in the previous test based on rDNA nucleotide (Fig. 2) sequence comparison; never-

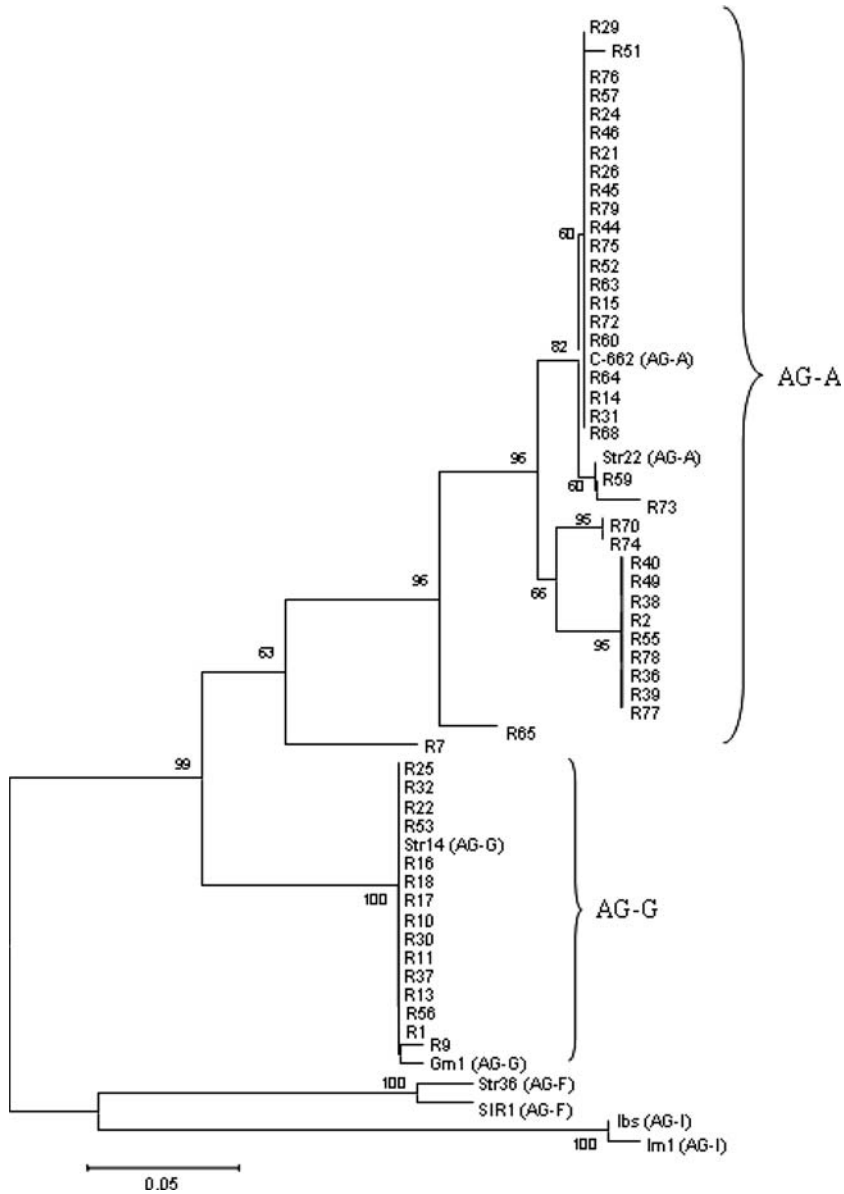

Fig. 2 Neighbour-joining tree generated from the comparison of ITS region nucleotide sequences of 51 binucleate *Rhizoctonia* isolates from strawberry and 8 previously characterized strains from GenBank (Table 1). Anastomosis groups are indicated by brackets to the right. Genetic distance is indicated by a bracket at the lower left

theless the Italian isolates did not cluster exactly with the correspondent AG testers used in the RFLP analysis. A good similarity was found between R15 and R29, belonging to AG-A, among AG-A tester Str22 and isolates R15 and R29, while R38 showed the highest similarity with the Am1 strain, the other AG-A tested isolates inserted in the analysis (Fig. 3). This analysis confirmed the wide variability observed within AG-A Italian isolates and the highest similarity among those identified as AG-G in the previous test based on the ITS sequence.

Analysis of molecular variability

AMOVA, carried out on hierarchic levels based on the geographic location, showed that molecular variations between north and south *Rhizoctonia* isolates accounted for 36.6% of the total variation but that most of the variation (61.1%) was within each of the growing areas, named as regions from where the isolates originated (Table 4). The isolate sample from northern Italy was represented by four haplotypes: 60% of them represented the haplotype corresponding to AG-

Table 3 Restriction fragment length polymorphism (RFLP) size of initial amplified fragments of tester isolates and 16 representative *Rhizoctonia* spp. isolates subjected to the analysis

Isolate	Fragment size (base pair)
R1	1400
R18	1400
R22	1400
R56	1400
R60	1400
R72	1400
Am1	1400
Gm1	1400
Str36	1400
R65	1800
R7	1800
Str 22	1400, 1800
R15	1400, 1600, 1800
R70	1400, 1600, 1800
R29	1400, 1600, 1800
Im1	1400, 1600, 1800
R38	1400, 3000
G14	1400, 3000
R77	3000
R55	3000

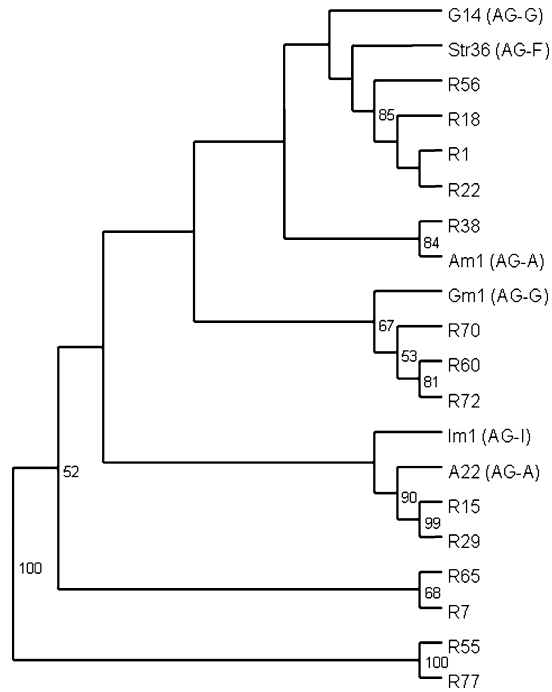

Fig. 3 Cluster analysis by unweighted pair-group method using arithmetic averages of fragment size of amplified portions of 28S rRNA digested individually with three restriction enzymes. Numbers at the nodes represent bootstrap generated by 1000 replications using FreeTree programme. Only values greater than 50% are indicated on the dendrogram

G isolates (Table 5, Fig. 2), and 35% was represented by two haplotypes belonging to the two widest sub-clusters of AG-A in the neighbour-joining tree (Table 5, Fig. 2). The isolates from the south were represented by eight haplotypes, seven of which (90%) belonged to AG-A isolates (Table 5, Fig. 2).

Discussion

All binucleate *Rhizoctonia* spp isolates from strawberry roots differed mainly in colony colour, and there was a not great difference in colony morphology and in plant host relationships; nevertheless a high genetic variability was observed.

Most of the isolates were pathogenic on strawberry, but they were able to produce necrosis on host plants belonging to botanical families other than the original host, thus showing a saprophytic behaviour. The pathogenicity of *Rhizoctonia* isolates on strawberry was very similar to that observed on faba bean (78% similarity), while their pathogenicity on strawberry differed strongly from that observed on wheat (13% similarity) and was far from that observed on brassica.

As several nutritional and abiotic factors are involved in the aggressive expression of *Rhizoctonia* spp. in strawberry black root rot disease (Huber and McCay-Buis, 1993; LaMondia, 2003; LaMondia and Martin 1989), artificial inoculations were performed to compare the pathogenicity of each isolate on strawberry and three herbaceous host plants. The pathogenicity test performed with colony disks revealed the capacity/incapacity of *Rhizoctonia* isolates to generate necrosis in a restricted portion of root. This would have been more difficult to obtain using the inoculum mixed in the soil. Considering that the pathogenicity tests were performed to allow the direct contact of *Rhizoctonia* colonies with host roots, the results showed a generally weak pathogenicity. Indeed no dead plants were observed but only a growth reduction in the most severe

Table 4 AMOVA statistics obtained for analysis of *Rhizoctonia* strains based on the geographic location of the isolates. Nucleotide sequence data handled as representative haplotype obtained from the ITS1 region

Type of variation	d.f.	Variance components	Percentage variation
Climatic areas (north and south Italy)	1	1.812	36.58
Within the same climatic area	2	.114	2.31
Within populations of four regions	47	3.027	61.11

plant-fungus relationships recorded. The study of strawberry *Rhizoctonia* spp. using rDNA ITS region sequence analysis as the main tool, made it possible to overcome the problems of strain characterization encountered earlier in our study due to the high affinity of hyphal cells among *Rhizoctonia* spp. isolates.

A good correspondence has still not been obtained within the *Ceratobasidium* genus between identification based on hyphal anastomosis groups and that based on rDNA ITS regions, as for *Thanatephorus* (Cubeta et al., 1991; Gonzales et al., 2001; Kuninaga et al., 1997; Sneh, Burpee, & Ogoshi, 1991). Nevertheless, in our study the neighbour-joining tree based on the ITS region sequence data clearly showed that *Rhizoctonia* spp. isolated from strawberry in Italy formed two clusters corresponding to AG-A and to AG-G (Fig. 3). The four isolates non-pathogenic on strawberry were AG-A while all AG-G isolates were pathogenic on strawberry. The phylogenetic tree based on nucleotide sequences and additional RFLP analysis showed a higher intra-group genetic variability of AG-A isolates than that observed for AG-G isolates. In this study, among the isolates identified as AG-A, isolates R70 and R74, analyzed in a phylogenetic tree together with all representative sequences of binucleate *Rhizoctonia* AGs available in GenBank, clustered with two isolates previously identified as AG-K (AF479017 and AB12145, isolated respectively from *Prunus communis* and *Allium cepa*), despite

Table 5 Haplotype frequency of *Rhizoctonia* isolates according to hierarchic subdivision based on climatic areas (south and north) and their relative frequency within each region of south

Haplotype[1]	Southern regions			SOUTH (Total 3 regions)	NORTH (1 region)
	Campania (5 isolates) %	Campania (17 isolates) %	Sardina (9 isolates) %	31 isolates %	20 isolate %
1	60.0	29.4		25.8	
2	20.0			3.2	
3		35.3	55.6	35.5	10.0
4		17.6		9.7	25.0
5		5.9	11.1	6.5	
6			22.2	6.5	
7			11.1	3.2	
8	20.0	11.8		9.6	60.0
9				0	5.0

[1] Isolates belonging to each representative haplotype

1: R2, R36, R39, R40, R49^{np}, R55, R77^{np}, R78^{np}

2: R38

3: R14, R29, R44^{np}, R45, R51, R68, R60, R63, R64, R72, R75, R76, R79

4: R15, R21, R24, R26, R31, R46, R52, R57

5: R74, R70

6: R59; R73^{np}

7: R65

8: R1, R9, R10, R11, R13, R16, R17, R18, R22, R25, R30, R32, R56, R37, R53

9: R7

^{np} : isolates not pathogenic on strawberry under artificial inoculation

maintaining their position within the main clade grouping AG-A isolates (data not shown). Given that AG-K representative isolates were not available, and their anastomosis reaction with the AG-A tester did not differ from that of other isolates identified as AG-A, the R70 and R74 isolates were considered as belonging to AG-A. Only two isolates, R65 and R7, were not well defined by the phylogenetic tree, even though their ITS region sequences were closer to those of AG-A. This illustrates the high variability observed within the group of isolates belonging to AG-A.

The isolate cluster obtained by UMPGA, based on the PCR-RFLP pattern of 28S rRNA (Fig. 3), corresponded to that obtained with the neighbour-joining tree based on nucleotide sequences (Fig. 2), but in this case the results were not so clearly defined. The RFLP analysis also confirmed the position of isolates R65 and R7 as separate from AG-A and AG-G.

Unlike results in the USA, where AG-A and AG-I have always been the most frequent anastomosis groups (Martin, 1988, 2000), Italian populations of strawberry binucleate *Rhizoctonia* seem to be AG-A and AG-G. This is similar to what was found in South Africa where *Rhizoctonia* spp. from strawberry belonged to AG-A and AG-G, with a low AG-I occurrence (Botha, Denman, Lamprecht, Mazzola, & Crous, 2003).

In Italy, variation within the isolate sample of each region accounted for a large amount (61%) of the total variation. Besides the high intra-group variability of AG-A isolates, AG-G and AG-A have almost always been isolated together in strawberry samples from the same location and the same region, which explains the high genetic variability found within each region. The variation observed (37%) between north and south climatic areas is due to the prevalence of AG-A strains in the south and of AG-G in north Italy and suggests that environmental conditions can affect the composition of the *Rhizoctonia* agent of strawberry black root rot. This hypothesis is supported by the findings of Martin (2000) who found AG-A to be the most frequent AG on the central Californian coast and the variation in the relative recovery frequency of other AGs between collection sites.

The availability of ITS region sequences and the recent increase in the number of strawberry *Rhizoctonia* binucleate isolates held in the GenBank database will be useful for further studies on the variability of binucleate *Rhizoctonia* isolated from strawberry, from different areas, countries and environments, and will make it possible to improve the knowledge about binucleate *Rhizoctonia* as the agent of strawberry black root rot. The possibility of misidentified fungal isolates in the GenBank does, however, still make it essential to use multiple methods, as in this study.

The genetic characteristics of *Rhizoctonia* isolates from strawberry in Italy, their weak pathogenic behaviour and low host specialization suggest that it is a genetically variable fungus, with saprobic behaviour that plays a role in strawberry black root rot.

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