European Journal of Plant Pathology **109:** 861–870, 2003.

Development of specific PCR primers for identification and detection of *Rhizopycnis vagum*

Stefano Ghignone[1], Giacomo Tamietti[2] and Mariangela Girlanda[1,3,*]
[1]*Dipartimento di Biologia Vegetale, Viale PA Mattioli 25, 10125 Torino, Italy;* [2]*Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy;* [3]*IPP sezione di Torino, Viale PA Mattioli 25, 10125 Torino, Italy; *Author for correspondence (Fax: ++39-011-6705962; E-mail: mariangela.girlanda@unito.it)*

Accepted 23 April 2003

Key words: dark sterile mycelia, PCR diagnostics, ribosomal DNA, vine collapse

Abstract

Rhizopycnis vagum is a recently described coelomycete known to belong to the complex of root rot pathogens contributing to vine decline of cucurbits in several parts of the world. However, the fungus has also been reported to infect tomato, and as an endophytic associate of mycorrhizal roots of wild, asymptomatic *Pinus halepensis* and *Rosmarinus officinalis* plants in Italy. To accelerate epidemiological and ecological investigations on this fungus, a PCR primer pair was developed. Primers Rv1-F and Rv1-R were designed, based on alignment of internal transcribed spacer (ITS) sequences (ITS1-5.8S-ITS2), which amplified a 396-bp fragment from all *R. vagum* isolates tested, including isolates pathogenic to melons and endophytic isolates from mycorrhizae. Specificity of the primer pair was verified both *in silico* (BLAST searches using each primer string as a query) and in PCR assays, where the primers failed to amplify DNA from any isolate of fungi taxonomically related to *R. vagum* (e.g. *Massarina walkeri* and *Stagonospora* spp.) and other vine decline and common soilborne pathogens (e.g. *Monasporascus cannonballus*, *Acremonium cucurbitacearum*, *Fusarium* spp. and *Rhizoctonia solani*). Under optimum conditions, detectable amplification of the specific sequence required 0.05 pg of target DNA. Amplification of the expected 369-bp fragment was also obtained from DNA root extracts of nearly asymptomatic *Cucumis melo* plants inoculated with *R. vagum* under greenhouse conditions.

Introduction

Rhizopycnis vagum D.F. Farr is a recently described coelomycetous fungus (gen. et sp. nov., Farr et al., 1998) known to contribute to 'vine decline' of cucurbits in several parts of the world. Vine decline (also referred to as sudden collapse or sudden death) comprises a group of diseases resulting in collapse of the vine, causing premature fruit ripening, reduced sugar accumulation and exposure of fruit to sunburn. Affected plants exhibit one or more additional symptoms, including xylem discoloration (vascular wilt), crown rot, root rot or phloem discoloration (Bruton 1998; Bruton et al., 1998). Vine decline is rarely caused by a single pathogenic organism; rather, it is often caused by a disease complex, although one pathogen may predominate. The main root rot agents are *Monasporascus cannonballus* Pollack and Uecker, *Acremonium cucurbitacearum* Alfaro-García, W. Gams and García-Jim., and *R. vagum*. Furthermore, numerous factors are involved in the occurrence and severity of this disease, amongst which temperature plays a major role in melon production areas where *M. cannonballus* and *A. cucurbitacearum* are present. Mediterranean regions appear particularly prone to the development and spread of the disease, since they are important cucurbit production areas and have hot and arid to semiarid climates.

Rhizopycnis vagum was first reported, as an unknown, *Stagonospora*-like fungus, and possibly

involved in the vine decline complex of cantaloupe (*Cucumis melo* var. *cantalupensis* Naudin) in the Rio Grande Valley of Texas (Mertely et al., 1991). In 1995 it was isolated from cantaloupe, honeydew (*C. melo* var. *indorus* Naudin) and watermelon (*Citrullus lanatus*) plants grown in a field in the Rio Grande Valley having a history of vine decline. In subsequent greenhouse tests these isolates were found to incite significant root necrosis, stunting and reduction in leaf area in inoculated cantaloupe (Miller et al., 1996). The same fungus was isolated from other cantaloupe-growing areas in Guatemala and Honduras (Bruton and Miller, 1997a–c) and in California from melon (*C. melo*) roots, where, along with *A. cucurbitacearum*, it was the most frequently isolated fungus (followed by *Pythium* spp., *Macrophomina phaseolina*, *Verticillium dahliae*, *Fusarium solani*, *M. cannonballus* and *Rhizoctonia solani*) (Gwinne et al., 1997; Aegerter et al., 2000). In the latter study, occurrence of these different fungi varied with root symptomology, *R. vagum* being most frequently associated with dry, corky root rot. Frequently, more than one fungus was isolated from a single lesion or root, and *A. cucurbitacearum* and *R. vagum* were often isolated together. In pathogenicity tests on melon, Californian isolates of *R. vagum* caused only slight root rot in field microplots, with a more severe secondary root rot occurring under greenhouse conditions (Aegerter et al., 2000). Between 1996 and 1999, *R. vagum* was detected as being associated with melon roots exhibiting pinkish discoloration from plants affected by vine decline collected in different Spanish production areas (Armengol et al., 2000). In 1997, vine collapse was observed in Italy in Emilia-Romagna region on *C. vulgaris* and *C. melo* plants growing in the greenhouse, and in 2001 on field-cultivated *C. melo* and *C. sativus* plants, where it reduced seed yield up to 50% (Montuschi, 2001).

Although primarily reported from melon roots, *R. vagum* appears associated with other, non-cucurbitaceous hosts. In Central and Southern Italy it was isolated from primary and secondary tomato (*Lycopersicon esculentum*) roots showing typical corky root symptoms, along with *Pyrenochaeta lycopersici*, the cause of tomato corky root (Porta-Puglia et al., 2001). *Saccharum officinarum* (sugarcane) may be considered another non-cucurbitaceous host of *R. vagum*. The fungus (erroneously identified as *Stagonospora subseriata*) was isolated, although with low frequency, from roots of poorly-developed canes (Watanabe, 1975; 1994; Farr et al., 1998). A somewhat different association was revealed by an investigation of fungal dark sterile endophytes of mycorrhizal roots of neighboring *Pinus halepensis* Mill. (ectomycorrhizal) and *Rosmarinus officinalis* (endomycorrhizal) plants in a wild Mediterranean forest in Northern Italy (Liguria). A sterile morphotype was consistently isolated from surface-sterilized mycorrhizae of both hosts in collections made over 11 years (1990–2001). Host plants had an healthy appearance and asymptomatic roots. The fungus remained sterile in spite of various and prolonged attempts to induce sporulation. Internal transcribed spacer (ITS) regions (ITS1-5.8S-ITS2) of representative isolates were, therefore, sequenced (AF373055, AF373054, AF373053, AF373052, AF373051) and, unexpectedly, found to be almost identical with the GenBank sequence of *R. vagum* (AF022786). Colony features and morphology of somatic structures (including characteristic, tuberculate dark chlamydospores) were also consistent with *R. vagum*, suggesting conspecificity (Girlanda et al., 2002).

Poorly understood etiology and increasing spread of vine declines correlated with infection by *R. vagum* emphasize a need for development of efficient and reliable tools for diagnosis. Furthermore, reports of the unexpected association of *R. vagum* with distantly-related hosts raise intriguing questions about the actual distribution of this fungus and its ecological potential. The PCR approach has proved valuable for detection of many soilborne pathogenic fungi. ITSs in rDNA are especially suited as targets, and their specific detection is possible with high sensitivity requiring only small amounts of template (Ghignone and Migheli, 2001). Specific ITS-based primers have already been developed for the other two main components of cucurbit root rot complex, *M. cannonballus* (Lovic et al., 1995) and *A. cucurbitacearum* (Martinez-Culebras et al., 2002). The objective of this study was to develop specific PCR primers for identification and detection of *R. vagum* in plant material.

Materials and methods

Fungal and plant material

Thirty-four *R. vagum* isolates were used, including reference isolates obtained from melon plants exhibiting vine decline symptoms in Arizona, California, Texas, Honduras and Costa Rica. These included the strain TX951120 (= ATCC 201387) designated as holotype (Farr et al., 1998). Other *R. vagum* isolates tested were

recovered as dark sterile endophytes of mycorrhizal roots of neighboring, asymptomatic *P. halepensis* and *R. officinalis* plants in a Mediterranean forest in Italy (Girlanda et al., 2002). The soilborne fungi tested were taxonomically and ecologically related to *R. vagum*, and included both other dark sterile endophytes obtained from *P. halepensis* and *R. officinalis* (Girlanda et al., 2002) and common vine decline pathogens. Isolates of *Phytophthora* spp., *Pythium ultimum*, *R. solani*, *Sclerotinia sclerotiorum, F. graminearum*, *F. oxysporum* f. sp. *melonis* were kindly provided by Prof. M.L. Gullino of Di.Va.P.R.A., University of Turin, Italy. Other fungal isolates were from the collection of Mycotheca Universitatis Taurinensis (MUT) and the Mycology Laboratory (CML) of Turin, Italy, or were obtained from the Centraalbureau voor Schimmelcultures (CBS) of Utrecht, the Netherlands. Stock cultures, stored at 4 °C, were maintained on malt extract agar (MEA), except *Phytophthora* and *Pythium* isolates which were cultured on vegetable eight agar (V8). Table 1 presents more detailed information for isolates used in the experiments shown in Figures 2–4.

Infected and non-infected roots of *C. melo* were obtained during pathogenicity assays in the greenhouse following the protocol of Aegerter et al. (2000). *C. melo* plants (cultivar Cantalupo di Charentais) were grown in 3-l pots filled with potting mix (40% washed sand, 20% nitrified redwood, 20% peat moss, 20% pumice rock vol/vol/vol/vol, and a trace of lime). Inoculum of *R. vagum* isolates CA951110, TX951120, MUT 2720, MUT 2722, CLM Ph020, CLM Ph028 or CLM Ro026, CLM Ro067 and CLM Ro076 was mixed with the medium at 5×10^3 CFU/g soil. Inoculated and non-inoculated roots were harvested after 28 and 50 days.

Table 1. Fungal species appearing in Figures 2–4, isolate code, origin and results of PCR amplification using universal (ITS1/ITS4) and *R. vagum*-specific (Rv1-F/Rv1-R) primers

Fungal species	Isolate	Origin	PCR analyses[1]	
			Rv1-F/Rv1-R	ITS1/ITS4
R. vagum D.F. Farr	AZ990313	Arizona	•	•
R. vagum D.F. Farr	HN010011	Honduras	•	•
R. vagum D.F. Farr	CA951110	California	•	•
R. vagum D.F. Farr	CA95112	California	•	•
R. vagum D.F. Farr	TX951120	Texas	•	•
R. vagum D.F. Farr	TX960103	Texas	•	•
Fungal endophyte Ph007 (*R. vagum*)	MUT 2720	Italy	•	•
Fungal endophyte Ph020 (*R. vagum*)	CLM Ph020	Italy	•	•
Fungal endophyte Ph066 (*R. vagum*)	MUT 585	Italy	•	•
Fungal endophyte Ph092 (*R. vagum*)	MUT 784	Italy	•	•
Fungal endophyte Ro026 (*R. vagum*)	CLM Ro026	Italy	•	•
Fungal endophyte Ro063 (*R. vagum*)	MUT 2722	Italy	•	•
Fungal endophyte Ro076 (*R. vagum*)	CLM Ro076	Italy	•	•
Fungal endophyte Ro109 (*R. vagum*)	MUT 2723	Italy	•	•
A. cucurbitacearum Alfaro-García, W. Gams and García-Jim.	CBS 525.93	Spain	○	•
D. melonis Beraha and M.J. O'Brien	CBS 507.78	Texas	○	•
D. bryoniae (Fuckel) Rhem	TX97128A	Texas	○	•
F. graminearum Schwabe	n.d.	Italy	○	•
F. oxysporum f.sp. *melonis* W.C. Snyder and H.N. Hansen	n.d.	Italy	○	•
F. semitectum Berk. and Ravenel	n.d.	Italy	○	•
M. phaseolina (Tassi) Goid.	CBS 461.70	Denmark	○	•
M. walkeri Shoemaker, C.E. Babc. and J.A.G. Irwin	CBS 257.93	Australia	○	•
M. cannonballus Pollack and Uecker	CBS 609.92	Arizona	○	•
Phytophthora sp.	n.d.	Italy	○	•
P. ultimum Trow	n.d.	Italy	○	•
R. solani J. G. Kühn	n.d.	Italy	○	•
S. sclerotiorum (Lib.) de Bary	n.d.	Italy	○	•
S. elegans (Berk.) Sacc. and Traverso	CBS 712.95	Netherlands	○	•
S. maculata (Grove) R. Sprague	CBS 284.69	Germany	○	•
V. albo-atrum Reinke and Berthold	MUT 331	Italy	○	•

[1]PCR amplification positive (•) or negative (○).

To verify colonization by the pathogen, 2-mm segments of representative root tissue were plated on 2% MEA, either after thorough washing in sterile water or washing followed by surface sterilization in 50% hypochlorite solution in ethyl alcohol. DNA was extracted (as described below) from both uninfected and roots colonized by the fungus.

DNA extraction

Fungal isolates were grown in 70 ml 2% malt extract liquid medium for 1–2 weeks at room temperature with rotary shaking. Mycelia were harvested by vacuum filtration through filter paper and immediately stored at −80 °C. DNA extractions from plant tissues of *C. melo* were performed on fresh leaves. Prior to extraction, leaf surfaces were disinfected by washing in a 1 : 10 solution of sodium hypochlorite, with three drops of Tween 20® added. Leaves were then rinsed twice in distilled water and air-dried for 30 min.

About 500 mg mycelia or leaves were frozen in liquid nitrogen, ground in a pre-chilled mortar and pestle and transferred to a 2.0-ml Eppendorf tube. DNA extractions were carried out following a CTAB-based extraction method. In brief, 800 µl of hot extraction buffer (2% CTAB, 0.1 mM Tris–HCl pH 9, 1.4 M NaCl, 20 mM EDTA pH 8, 0.2% β-mercaptoethanol, 2% PVP) were added to the powdered leaves or mycelium, vortexed and incubated at 60 °C for 60 min. After 10-min centrifugation at 25,000× *g*, the aqueous phase was removed to a new tube, and extracted twice with one volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1), followed by an extraction with an equal volume of chloroform. Nucleic acids were precipitated from aqueous phase by addition of one volume of cold isopropanol and incubated overnight at −20 °C. DNA pellets were collected by centrifugation at 25,000× *g* for 20 min; the DNA pellets were then washed with 70% ethanol, dried briefly, resuspended in 100 µl of sterile double-distilled water and stored at −20 °C until use. DNA extractions were diluted 1 : 150 and used as template for PCR amplification. DNA extraction from roots was performed with DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), following manufacturer's instructions.

ITS amplification and sequencing

Universal primers ITS1 (TCCGTAGGTGAACCT GCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990) were used to amplify the ITS regions between the small (18S gene) and large (28S gene) nuclear rDNA, including the 5.8S rDNA, of all fungal isolates tested. Amplifications were carried out in 50 µl volumes containing 30.5 µl of sterile double-distilled water, 5 µl of 10× Red*Taq* buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 11 mM $MgCl_2$ and 0.1% gelatin), 2 µl of dNTP stock (2.5 mM each of dATP, dCTP, dGTP, dTTP; Sigma-Aldrich, St. Louis, MI, USA), 3 µl of each oligonucleotide primer (0.016 mM), 1 U of Red*Taq* DNA polymerase (Sigma-Aldrich), and 5 µl of the DNA template. Each set of amplification included a negative control in which 5 µl of sterile water was substituted for the DNA template. PCR reactions were performed by a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Cycling parameters for the amplification reaction were: initial denaturation for 5 min at 94 °C; 40 cycles of 40 s of denaturation at 94 °C, 45 s of annealing at 55 °C and 1.30 min of extension at 72 °C, final extension for 7 min at 72 °C. Five microliter of PCR product were checked by separation in a 1% TAE agarose gel (Sambrook and Russel, 2000) stained with 0.5 µg/ml ethidium bromide and viewed with ultraviolet light.

Aliquots of about 3 µg of DNA, purified from a gel with Qiaex II Kit (Qiagen), were used for sequencing. The complete ITS regions and the 5′ region of the 18S rDNA subunit were directly sequenced in both directions by the 'Service d'analyse et de synthèse' at Université de Laval, Québec, Canada.

Primer design

Sequences of both DNA strands of the ITS region were aligned for each *R. vagum* Italian isolate (MUT 585, MUT 784, MUT 2720, MUT 2721 and MUT 2723) and manually adjusted using GeneDoc v. 2.6 (Nicholas and Nicholas, 1997). The adjusted sequences of the ITS plus strands were then aligned to obtain a consensus sequence, which was analyzed with the Primer3 software (Rozen and Skaletsky, 1998), which sets oligonucleotide melting temperature, size, G-C content, PCR product size, positional constraints within the source sequence. Parameters were set which identified the best primer pair to yield an amplicon of about 400 bp at a melting temperature >60 °C. This ensured the stringency needed for the highest specificity. All suggested primer pairs were subjected to BLAST searches to confirm their specificity for *R. vagum* AF022786.

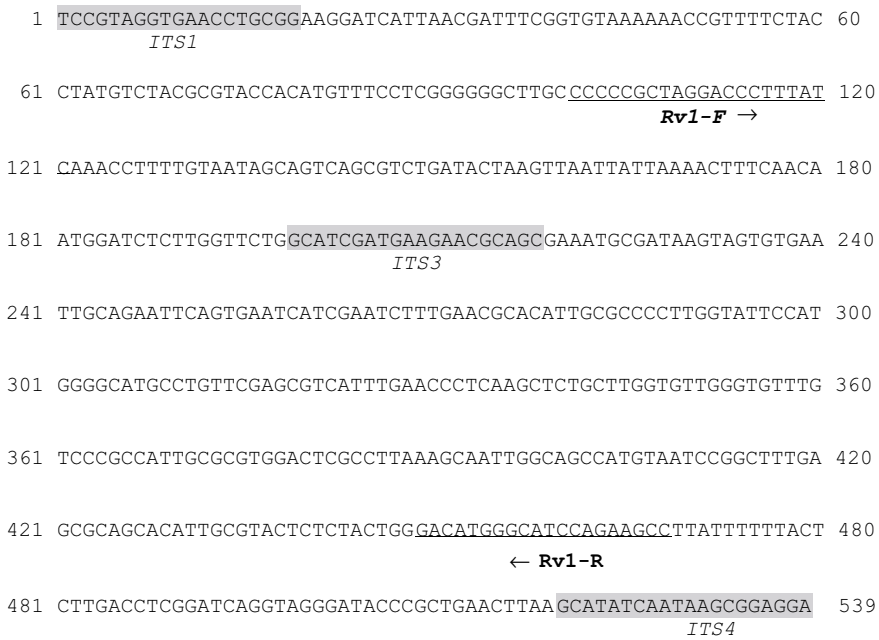

Figure 1. 5′-3′ sequence of the ITS region (ITS1-5.8S-ITS2) of *R. vagum* AF022786, showing position of the primer pair Rv1-F and Rv1-R (underlined) with respect to the universal primers ITS1, ITS3 and ITS4 (shaded).

A selected pair of primers, named Rv1-F and Rv1-R (Figure 1), were custom synthesized by Sigma-Genosys (Cambridge, UK).

Primer specificity and sensitivity tests

The primer pair Rv1-F/Rv1-R was tested for amplification of the predicted size fragment, specificity and sensitivity. After preliminary trials to optimize annealing temperature, the following protocol was chosen: initial 5 min denaturation at 94 °C; 27 cycles of 40 s denaturation at 94 °C, 50 s annealing at 64 °C, 50 s extension at 72 °C; and 7 min final extension at 72 °C. Products (15 µl) were analyzed by electrophoresis in a 1.2% TAE agarose gel, stained with 0.5 µg/ml ethidium bromide and visualized by ultraviolet light.

Primer specificity was tested against isolates of soilborne fungi taxonomically and ecologically related to *R. vagum*. Fungal DNA was also amplified with the universal primers ITS1 and ITS4, to avoid false negatives. Furthermore, specificity was tested using as target *C. melo* DNA. Plant DNA was also amplified with the primer pair B48557/A49291 (Taberlet et al., 1991), specific for a non-coding region of the chloroplast DNA. PCR amplifications from *C. melo* roots infected by *R. vagum* isolates TX951120, CA951110, MUT 2720, MUT 2722, CLM Ph020, CLM Ro026 and CLM Ro076 were performed using 5 µl DNA as template.

The minimum amount of fungal DNA detectable by PCR was evaluated performing reactions with 10-fold serial dilution of ITS PCR products of isolate MUT 784, amplified with universal primers ITS1 and ITS4, ranging from 10 ng to 1 fg.

Results

Primer pair selection

A pair of oligonucleotides, the 21-bp long Rv1-F (CCCCCGCTAGGACCCTTTATC) and the 20-bp long Rv1-R (GGCTTCTGGATGCCCATGTC), were designed from the consensus sequence of the alignment of the ITS sequences of isolates MUT 585, MUT 784, MUT 2720, MUT 2721 and MUT 2723. These sequences shared 98.8–99.4% similarity with the previously published sequence of *R. vagum* (AF022786). The predicted size of the amplicon was 369 bp. The positions and orientations of primers Rv1-F and Rv1-R in the ITS region, with respect to the universal primers ITS1, ITS3 and ITS4, are shown in Figure 1.

In silico primer pair specificity was evaluated searching the NCBI database, submitting primer strings separately. The BLAST search with the 21-bp string of primer Rv1-F as query retrieved as the most similar fungal sequences the published sequences of the endophytic *R. vagum* isolates (AF373055, AF373054, AF373053, AF373052, AF373051) and *R. vagum* AF022786 (100% identity on 21 bp stretches, scores 42, e-values 1e-04); other relevant alignments were found with sequences of *Umbrinosphaeria caesariata* AF261069 (100% identity on 14 bp stretch, score 28, e-value 2.0) and *Massarina walkeri* AF383965 (100% identity on 13 bp stretch, score 26, e-value 7.7). The BLAST search with the 20-bp string of primer Rv1-R as query also retrieved as the most similar fungal sequences the published sequences of the endophytic *R. vagum* isolates (AF373055, AF373054, AF373053, AF373052, AF373051) and *R. vagum* AF022786 (100% identity on 20 bp stretches, scores 40, e-values 5e-04); other relevant alignments were found to sequences of an unidentified leaf litter ascomycete coded AF502865 (94% identity on a 19 bp stretch, score 30, e-value 0.5) and *Leptosphaeria typharum* AF439465 (100% identity on a 15 bp stretch, score 30, e-value 0.5). Except for the expected target sequences, no other BLAST results were common to both searches, confirming specificity of the primer pair.

Amplification with specific primers

Prior to primer specificity tests, the integrity of all genomic DNAs of fungal isolates and plant samples was checked for amplification with ITS universal ITS1/ITS4 primers and primers B48557/A49291 (specific for a non-coding region of the chloroplast DNA) respectively.

The primer pair Rv1-F and Rv1-R successfully amplified target DNA from both pathogenic and endophytic *R. vagum* isolates (21 and 12 isolates respectively). PCR amplification resulted in all cases in the predicted single band of 369 bp. No reaction product was generated following amplification of DNA from any other fungi tested (Table 1, Figure 2b). No amplification product was obtained with genomic DNA of plant samples of *C. melo*.

The sensitivity assay on isolate MUT 784 allowed detection of up to 0.05 pg of the ITS target sequence (Figure 3). Assuming a rDNA repeat copy number of 100–200 (as in *Saccharomyces caerevisiae*, Johnston et al., 1997), such a minimal detectable amount would correspond to 40–80 genomes.

Detection of R. vagum

Inoculated *C. melo* exhibited only limited symptoms after 28 and 50 days (slight, light brown general root discoloration). However, *R. vagum* could be re-isolated from the inoculated plants. By using the rapid extraction protocol fragments of the expected 369 bp size were consistently amplified from total nucleic acids from the infected root systems of plants of both ages (Figure 4). Amplification was generally stronger for the older plants, consistent with higher root colonization by the fungus. However, PCR bands amplified from plant tissues were usually not as strong as those obtained from mycelia, suggesting the presence of PCR-inhibiting substances in plant material. Fungus-free root samples gave no amplification product.

Discussion

Root rot and associated vine decline (collapse) of cucurbits is due to a complex of pathogenic fungi, mainly comprising *M. cannonballus*, *A. cucurbitacearum* and *R. vagum*. The role of each fungal species may vary, depending on temperature (Bruton et al., 1999), host species (Porta-Puglia et al., 2001), root type and isolate virulence (Biernacki and Bruton, 2001). Similarity of symptoms induced by the different pathogen species and/or isolates may confound diagnosis based on symptom expression, creating a need for alternative detection methods. Specific, ITS-based PCR primers for *M. cannonballus* and *A. cucurbitacearum* have already been designed (Lovic et al., 1995; Martinez-Culebras et al., 2002), and in the current work were developed for *R. vagum*.

Characterization of *R. vagum* ITS region had been carried out by Girlanda et al. (2002). ITS (ITS1-5.8S-ITS2) regions of six isolates (comprising five endophytic isolates from *P. halepensis* and *R. officinalis* in Italy and isolate TX951120 from cantaloupe in the Rio Grande Valley, Texas) shared 98.4–100% sequence identity (over 502 bp stretches), and clustered together in phylogenetic analyses performed on a dataset comprising other sequences from anamorphic and teleomorphic taxa retrieved in BLAST searches. When first named, *R. vagum* was said to have

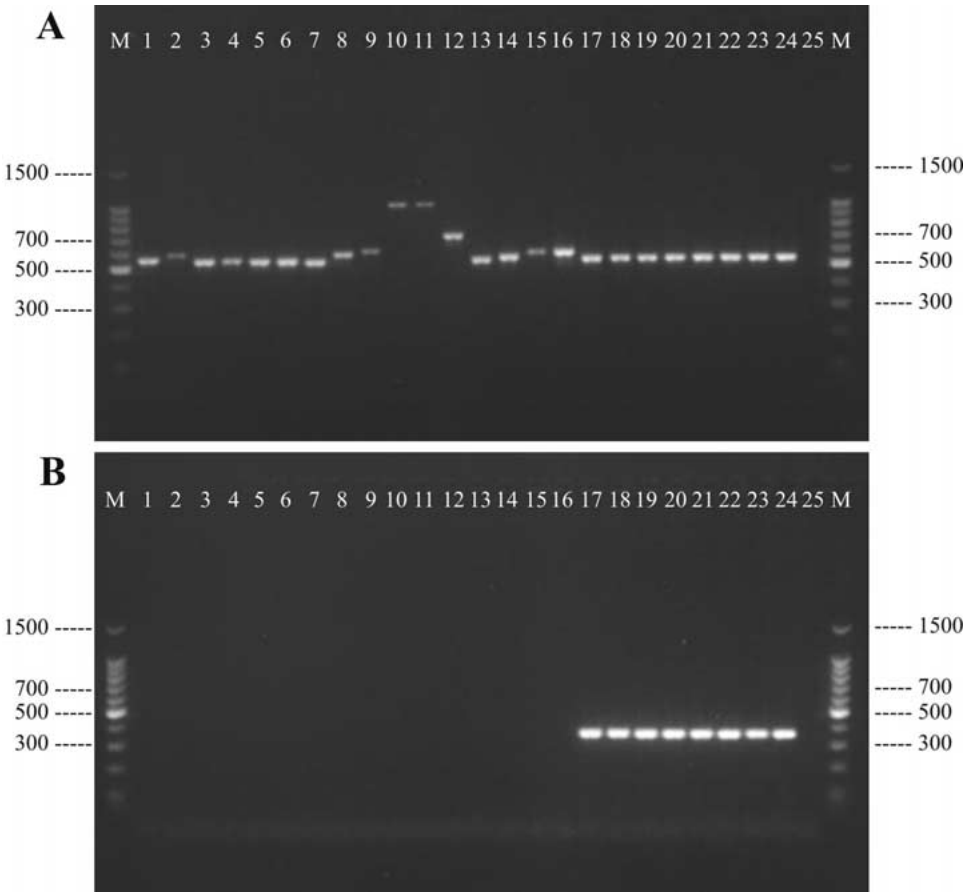

Figure 2. PCR amplification of fungal strain DNA using universal (ITS1/ITS4, (A)) and *R. vagum*-specific (Rv1-F/Rv1-R, (B)) primer pairs. PCR products were resolved by 1.5% agarose gel electrophoresis. Lane 1, *A. cucurbitacearum* CBS 525.93; lane 2, *D. melonis* CBS 507.78; lane 3, *D. bryoniae* TX97128A; lane 4, *F. graminearum*; lane 5, *F. oxysporum* f.sp. *melonis*; lane 6, *F. semitectum*; lane 7, *M. walkeri* CBS 257.93; lane 8, *M. phaseolina* CBS 461.70; lane 9, *M. cannonballus* CBS 609.92; lane 10, *Phytophthora* sp.; lane 11, *P. ultimum*; lane 12, *R. solani*; lane 13, *S. sclerotiorum*; lane 14, *S. elegans* CBS 712.95; lane 15, *S. maculata* CBS 284.69; lane 16, *V. albo-atrum*; lane 17, *R. vagum* MUT 585; lane 18, *R. vagum* MUT 784; lane 19, *R. vagum* MUT 2720; lane 20, *R. vagum* MUT 2723; lane 21, *R. vagum* AZ990313; lane 22, *R. vagum* HN010011; lane 23, *R. vagum* CA95112; lane 24, *R. vagum* TX960103; lane 25, water. M, 100 bp DNA ladder (Promega), fragment size in bp.

unknown phylogenetic affinities, but it was indicated to be morphologically most similar to *Stagonospora*, another coelomycetous fungus (Farr et al., 1998). In the study by Girlanda et al. (2002), *R. vagum* ITS regions shared good similarity (92–94% over 472 bp stretches) with *M. walkeri* (AF383965), a fungus belonging to the family *Lophiostomataceae*, order *Pleosporales*, which appeared to bear a sister-group relationship with *R. vagum* in phylogenetic analyses. In a study aimed at determining phylogenetic relationships and teleomorphic state of *Septoria passerinii* based on ITS sequences, Goodwin and Zismann (2001) suggested a close relationship between *R. vagum* and *Ophiosphaerella* spp. (family *Phaeosphaeriaceae*, order *Pleosporales*). However, their dataset did not include *M. walkeri*. In both studies, however, *R. vagum* appeared to be rather distantly related to *Stagonospora* spp. The primer pair Rv1-F/Rv1-R successfully amplified the ITS region from *R. vagum*, but not from the taxonomically-related fungi (e.g. *M. walkeri*, *Stagonospora* spp.). This was confirmed by *in silico* BLAST analysis using each primer as a query. The two primers were also selective against an array of common soilborne pathogens (including other vine decline fungi such as *M. cannonballus*, *A. cucurbitacearum*, *Didymella*

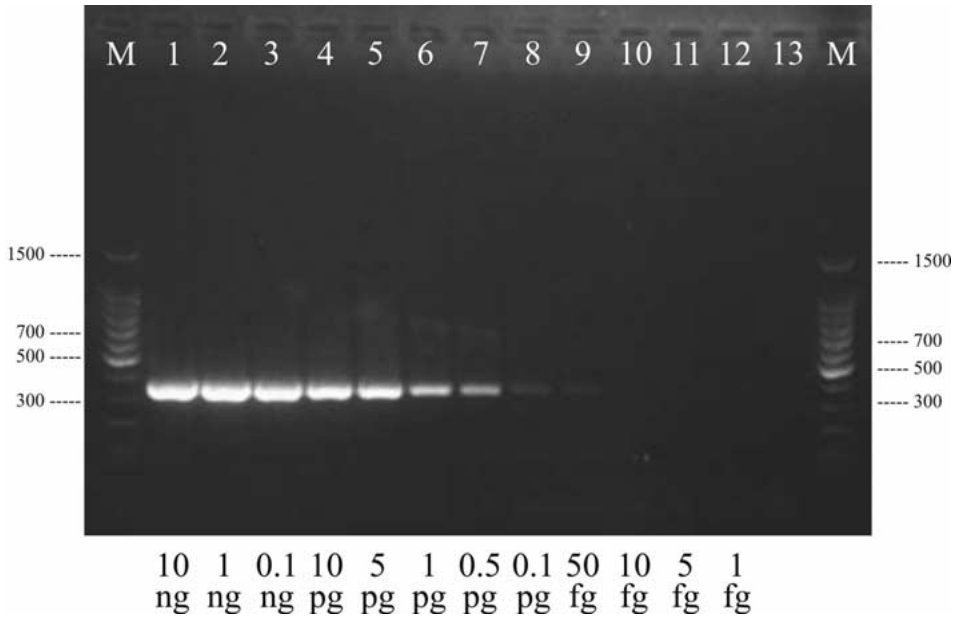

Figure 3. PCR amplification of decreasing amounts (ranging from 10^4 to 10^{-3} pg, Lanes 1–12) of target ITS sequence of *R. vagum* MUT 784 using the specific Rv1-F/Rv1-R primer pair. PCR products were resolved by 1.5% agarose gel electrophoresis. Lane 13, water. M, 100 bp DNA ladder (Promega), fragment size in bp.

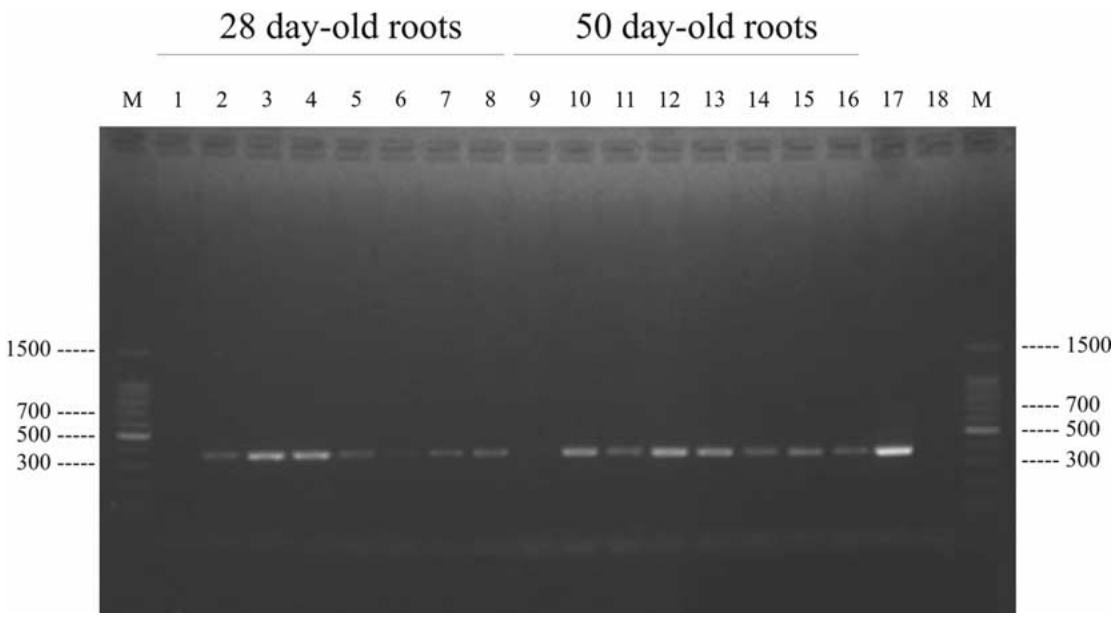

Figure 4. Detection of *R. vagum* in the roots of 28- and 50-day-old *C. melo* plants, artificially inoculated with *R. vagum* strains. DNA was extracted using DNeasy® Plant Mini Kit (Qiagen). PCR amplification was carried out using the specific Rv1-F/Rv1-R primer pair. PCR products were resolved by 1.5% agarose gel electrophoresis. Lanes 1 and 9, uninoculated plants; lanes 2–8 and 10–16, plants inoculated with *R. vagum* (lanes 2 and 10, TX951120; lanes 3 and 11, CA951110; lanes 4 and 12, MUT 2722; lanes 5 and 13, CLM Ph020; lanes 6 and 14, CLM Ro076; lanes 7 and 15, CLM Ro026; lanes 8 and 16, MUT 2720; lane 17, control DNA from *R. vagum* MUT 585; lane 18, water. M, 100 bp DNA ladder (Promega), fragment size in bp.

bryoniae, *M. phaseolina*, *Diaporthe melonis*), as well as other dark sterile fungi ecologically related to *R. vagum* and from the two hosts *P. halepensis* and *R. officinalis*. Sensitivity was good, as indicated by amplification with as little as 0.05 pg DNA, and from nearly asymptomatic melon roots. Primers Rv1-F/Rv1-R represent, therefore, a convenient diagnostic tool complementary or alternative to either inspection of symptoms or the isolation, culture-dependent approach. This is particularly suitable in a fungus, such as *R. vagum*, which produces similar symptoms to those caused by other pathogens, but sporulates scarcely.

Although a few pycnidia may be observed occasionally on symptomatic roots incubated in a moist chamber (Aegerter et al., 2000), or on sterilized roots of melons or stems of *Medicago* and *Stokesia* plated onto water agar (Farr et al., 1998), sporulation is scarce or absent on other cultural media (Armengol et al., 2000). Sporulation was never observed for endophytic isolates from *P. halepensis* and *R. officinalis* (Girlanda et al., 2002). Primers Rv1-F/Rv1-R amplified efficiently *R. vagum* DNA both from isolates pathogenic on melon and endophytic isolates from other, asymptomatic hosts (*P. halepensis* and *R. officinalis*); they should therefore prove beneficial for further epidemiological studies.

Although it is well known that many pathogens of economically-important crops may be endophytic in weeds (Sinclair and Cerkauskas, 1996), association of *R. vagum* with such distantly-related hosts as cucurbit crops and wild plants of *P. halepensis* appears intriguing. Dark sterile fungi are widespread root endophytes of mycorrhizal and non-mycorrhizal plants (co-existing, in the former hosts, with the 'official' mycorrhizal fungi) (Jumpponen and Trappe, 1998). These fungal associations are receiving increasing consideration, not only because of their ubiquitous presence and lack of host specificity, suggesting an important role in natural ecosystems (Jumpponen and Trappe, 1998), but also because they appear capable, at least in some cases, of enhancing host mineral nutrition and growth (Shivanna et al., 1994; Fernando and Currah, 1996; Jumpponen et al., 1998). Consequently they are regarded as non-conventional mycorrhizal symbionts (Jumpponen, 2001). Application of PCR-based methods, coupled with population genetic studies and further pathogenicity assays on several hosts may help unravel biological plasticity of *R. vagum*, allowing deeper understanding of the environmental risk associated with large-scale distribution of this fungus.

Acknowledgements

The authors gratefully acknowledge Prof. Anna Maria Luppi for the many comments to improve the manuscript, advice and support. Appreciation is also expressed to Prof. Quirico Migheli for critically reviewing the manuscript and helpful suggestions. Furthermore, authors are indebted to Dr. Benny Bruton and Prof. Ludovica Gullino for sharing fungal cultures. Research supported by grant 'ex-60%' 2002 Univ. Torino and by IPP-sez. Torino.

References

Aegerter BJ, Gordon TR and Davis RM (2000) Occurrence and pathogenicity of fungi associated with melon root rot and vine decline in California. Plant Disease 84: 224–230

Armengol J, Pellicer I, Vicent A, Bruton BD and García-Jiménez J (2000) *Rhizopycnis vagum* DF Farr, un nuevo Coelomycete asociato a raíces de planta de melón con síntomas de colapso en España. Boletín de Sanidad Vegetal Plagas (1° Trimestre) 26: 103–112

Biernacki M and Bruton BD (2001) Quantitative response of *Cucumis melo* inoculated with root rot pathogens. Plant Disease 85: 65–70

Bruton BD (1998) Soilborne diseases in Cucurbitaceae: Pathogen virulence and host resistance. In: McCreight J (ed) Cucurbitaceae '98 (pp 143–166) American Society for Horticultural Science Press, Alexandria

Bruton BD and Miller ME (1997a) Occurrence of vine decline diseases of muskmelon in Guatemala. Plant Disease 81: 694

Bruton BD and Miller ME (1997b) Occurrence of vine decline diseases of melons in Honduras. Plant Disease 81: 696

Bruton BD and Miller ME (1997c) The impact of vine declines on muskmelon production in Central America. Phytopathology 88: S120

Bruton BD, García-Jiménez J and Armengol J (1999) Analysis of the relationship between temperature and vine declines caused by *Acremonium cucurbitacearum* and *Monosporascus cannonballus* on muskmelon. Subtropical Plant Science 51: 23–28

Bruton BD, Russo VM, García-Jiménez J and Miller ME (1998) Carbohydrate partitioning, cultural practices, and vine decline diseases of Cucurbits. In: McCreight J (ed) Cucurbitaceae '98 (pp 189–200) American Society for Horticultural Science Press, Alexandria

Farr DF, Miller ME and Bruton BD (1998) *Rhizopycnis vagum* gen et sp nov, a new coelomycetous fungus from roots of melons and sugarcane. Mycologia 90: 290–296

Fernando AA and Currah RS (1996) A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (Fungi Imperfecti) on the growth of some subalpine plants in culture. Canadian Journal of Botany 74: 1071–1078

Ghignone S and Migheli Q (2001) Nuovi strumenti di diagnosi per funghi fitopatogeni. Informatore Fitopatologico 51: 7–22

Girlanda M, Ghignone S and Luppi AM (2002) Diversity of root-associated fungi of two Mediterranean plants. New Phytologist 155: 481–498

Goodwin SB and Zismann VL (2001) Phylogenetic analyses of the ITS region of ribosomal DNA reveal that *Septoria passerinii* from barley is closely related to the wheat pathogen *Mycosphaerella graminicola.* Mycologia 93: 934–946

Gwinne BJ, Davis RM and Gordon TR (1997) Occurrence and pathogenicity of fungi associated with melon vine decline in California. Phytopathology 87: S37

Johnston M, Hillier L, Riles L, Albermann K, André B, Ansorge W, Benes V, Brückner M et al. (1997) The nucleotide sequence of *Saccharomyces cerevisiae* chromosome XII. Nature 387(6632 Suppl): 87–90

Jumpponen A (2001) Dark septate endophytes – are they mycorrhizal? Mycorrhiza 11: 207–211

Jumpponen A and Trappe JM (1998) Dark septate endophytes: A review of facultative biotropic root-colonizing fungi. New Phytologist 140: 295–310

Jumpponen A, Mattson KG and Trappe JM (1998) Mycorrhizal functioning of *Phialocephala fortinii*: Interactions with soil nitrogen and organic matter. Mycorrhiza 7: 261–265

Lovic BR, Martyn RD and Miller ME (1995) Sequence analyses of the ITS regions of rDNA in *Monosporascus* spp to evaluate its potential for PCR-mediated detection. Phytopathology 85: 655–661

Martínez-Culebras PV, Abad P, Bruton B and García-Jiménez J (2002) Phylogenetic position of *Acremonium cucurbitacearum* and PCR primers for its identification (abstract). 6th European Conference on Fungal Genetics, 6–9 April 2002, Pisa, Italy

Merteley JC, Martyn RD, Miller ME and Bruton BD (1991) Role of *Monosporascus cannonballus* and other fungi in a root rot/vine decline of muskmelon. Plant Disease 75: 1133–1137

Miller ME, Bruton BD and Farr DF (1996) Association of a *Stagonospora*-like fungus on roots of melons exhibiting vine decline symptoms. Phytopathology 86: S3

Montuschi C (2001) Collasso delle cucurbitacee: Una malattia da indagare. *Agricoltura* 30: 31–33

Nicholas KB and Nicholas HB Jr (1997) GeneDoc: Analysis and Visualization of Genetic Variation http://wwwpscedu/biomed/genedoc/

Porta-Puglia A, Pucci N, Di Gianbattista G and Infantino A (2001) First report of *Rhizopycnis vagum* associated with tomato roots in Italy. Plant Disease 85: 1210

Rozen S and Skaletsky HJ (1998) Primer3. http://www-genomewimitedu/genome_software/other/primer3html

Sambrook J and Russel DW (2000) Molecular Cloning: A Laboratory Manual, 3rd edn, Vol 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Shivanna MB, Meera MS and Hyakumachi M (1994) Sterile fungi from zoysiagrass rhizosphere as plant growth promoters in spring wheat. Canadian Journal of Microbiology 40: 637–644

Sinclair JB and Cerkauskas RF (1996) Latent infection vs. endophytic colonization by fungi. In: Redlin SC and Carris LM (eds) Endophytic Fungi in Grasses and Woody Plants (pp 3–29) APS Press, St Paul

Taberlet P, Gielly L, Pautou G and Bouvet J (1991) Universal primer for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17: 1105–1109

Watanabe T (1975) Fungi isolated from the underground parts of sugarcane in relation to the poor ratooning in Taiwan (4). Coelomycetes. Transactions of The Mycological Society of Japan 16: 28–35

Watanabe T (1994) Pictorial Atlas of Soil and Seed Fungi. Morphologies of Cultured Fungi and Key to Species. Lewis, Boca Raton, Florida

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