Detection and Management of Downy Mildew in Rose Rootstock

B. J. Aegerter, Department of Plant Pathology, University of California, Davis 95616; **J. J. Nuñez**, University of California Cooperative Extension, Kern County, Bakersfield 93307; and **R. M. Davis**, Department of Plant Pathology, University of California, Davis

ABSTRACT

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A technique utilizing the polymerase chain reaction (PCR) was developed to investigate the occurrence and location of *Peronospora sparsa* in dormant, woody rose tissues. PCR primers were designed to amplify the internal transcribed spacer region of the ribosomal DNA of the pathogen. Inhibition of the reaction by plant compounds was minimized by optimizing the reagents used in the extraction of DNA from roses and in the amplification reaction. The PCR assay was capable of detecting as little as 2 pg of DNA from *P. sparsa* against a background of 4 ng of DNA from rose cane cortex. With this method, DNA of *P. sparsa* was detected in the cortex of stem and root tissues of symptomatic plants. Pathogen DNA also was detected in the cortex of crown tissues of asymptomatic mother plants used as a source of propagation materials. Epifluorescent and differential interference contrast microscopy were used to confirm the presence of abundant hyphae and oospores within the stem cortex of infected canes. Preplant treatments of dormant rootstock cuttings in fungicides or hot water were evaluated during natural outbreaks of the disease in commercial rose nurseries. In three trials conducted over 2 years, a 10-min preplant dip in the systemic fungicides metalaxyl or mefenoxam at rates of 100 to 10,000 mg a.i./liter reduced the area under the disease progress curve by 63 to 76% relative to nontreated plots. The evidence from PCR assays, microscopy, and fungicide trials all support the occurrence of perennating infections of *P. sparsa* within rose.

More than 50% of the bare-root roses produced in the United States are grown near Wasco, in Kern County, CA. In 2000, over 23 million rose plants were harvested at a farm-gate value of \$46 million (10). Since the early 1990s, this nursery production system has had a sporadic but continuing problem with rose downy mildew, caused by the oomycete *Peronospora sparsa*. Infected leaves quickly abscise, often resulting in severe defoliation and reduced plant vigor. During the first 2 months after budbreak, rootstock cuttings that are defoliated by downy mildew may fail to root, resulting in reduced stands. On older bushes, the reduced vigor can result in failure of the canes to reach the diameter required for the top grade and such plants often are never harvested. Dieback of young canes has been observed when the stems are severely diseased, presumably due to invasion by secondary pathogens such as *Botrytis* spp.

Downy mildew currently is managed with frequent preventative applications of

foliar fungicides after the plants begin to grow in January or February. Such applications are costly in terms of materials and labor, and can be difficult to undertake during rainy weather. Even when fungicides are applied, growers find the currently registered materials only marginally effective. Difficulties in disease control are compounded because the source of initial inoculum is unknown. Production fields are fumigated with methyl bromide prior to planting; therefore, soilborne inoculum seems an improbable source. Caneberries (*Rubus* spp.), which are alternative hosts of *P. sparsa*, are not cultivated in this production area, and infected wild hosts have not been observed in Kern County.

Because roses are vegetatively propagated, it has been suggested that mycelium of *P. sparsa* overwinters in rose propagation stock (5), but such transmission has never been demonstrated. In the bare-root rose industry in Kern County, rootstock is propagated by cuttings taken from blocks of perennial mother plants and the scion is propagated by buds collected from production fields. To avoid viruses, growers periodically return to material from Foundation Plant Material Services at the University of California (UC) at Davis. Rootstock cuttings are obtained from mother plants in the fall and typically planted in November. These cuttings root in the field and begin growing in January or February, depending on the planting date and seasonal temperatures. In some years, downy mildew ap-

pears on these cuttings just as they emerge from dormancy and this is typically the first downy mildew in a season. In the past, this was a particular problem with the rootstock Manetti's rose (*Rosa* × *noisettiana* Thory cv. Manetti). These observations led us to suspect that at least one source of initial inoculum may be within the rootstock cuttings.

In addition to concerns about sources of the pathogen within propagation materials, there is also the potential of exportation of infected plants to other areas. In the absence of symptoms, however, there is currently no means by which growers can ascertain if plants might be latently infected. Microscopic examination, while useful, is limited to a small volume of plant material and lacks specificity because the mycelial morphology of various fungi is similar. DNA-based methods, such as the polymerase chain reaction (PCR), are particularly suitable for the detection of obligate parasites like downy mildews.

Toward development of an integrated control program for rose downy mildew, we initiated investigations into the potential for infected stems to serve as sources of initial inoculum. The specific objectives of this research were to (i) develop and implement a PCR-based method for the detection of *P. sparsa* in woody rose tissues, (ii) examine survival structures within rose tissues by epifluorescent microscopy, and (iii) document the efficacy of fungicidal or hot-water dips of rootstock propagation wood in the control of epidemics of downy mildew in rose nurseries.

MATERIALS AND METHODS

Development of PCR-based detection assay. The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) was chosen as a target for amplification of *P. sparsa* DNA. The ITS has sequence variation at the interspecific level but low levels of intraspecific variation (11).

To obtain DNA for amplification and sequencing, symptomatic leaves collected from commercial fields in Kern County, CA were placed in a plastic box overnight at room temperature and 100% relative humidity to induce sporulation. Under a dissecting microscope, roughly 200 sporangiophores bearing sporangia were removed from the leaves with sterile jeweler's forceps and placed into a 1.5-ml microcentrifuge tube containing 100 µl of PCR buffer (undiluted GeneAmp10X PCR

Corresponding author: B. J. Aegerter
E-mail: bjaegerter@ucdavis.edu

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buffer II; Applied Biosystems, Foster City, CA). The suspension was sonicated with a Vibra Cell sonicator (Model VC50; Sonic & Materials Inc., Danbury, CT) using a pulse of 2 s duration delivering 39 W at the probe tip. The tubes then were centrifuged at maximum speed ($16,000 \times g$) for 5 s to pellet the cellular material. DNA was stored at -20°C prior to use in PCR.

PCR amplification of ITS1 and ITS2 and the 5.8S RNA gene of the rDNA was accomplished with the oligonucleotide primers ITS4 and ITS5 (24). The PCR was performed in 50- μl reactions containing 2.5 mM MgCl_2 , 200 μM of each deoxynucleoside triphosphate, 0.1 μM of each oligonucleotide primer, and 1 unit of Taq polymerase in 1 $\times$ GeneAmp PCR buffer II (Applied Biosystems). To this mixture, 1 to 3 μl of sonicated sporangial suspension was added. The reaction was carried out in a thermal cycler (EasyCycler, Ericomp Inc., San Diego, CA) programmed for 40 cycles, each consisting of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 2.5 min, with a single final extension at 72°C for 10 min.

Due to difficulties in obtaining satisfactory sequence data from the PCR product, the amplification fragment from four different isolates from Kern County was cloned into a plasmid in *Escherichia coli* with the TA cloning system (TOPO TA, Invitrogen Corp., Carlsbad, CA) using the manufacturer's recommendations. Successful transformants transferred to 10 ml of Luria-Bertani broth (13) were grown for 24 h on a shaker at 37°C . Plasmid DNA was extracted from *E. coli* with the QIAprep Spin Mini-prep Kit (Qiagen Inc., Valencia, CA). The amplification fragment was cut from the plasmid vector with the restriction enzyme *EcoRI*. These cloned fragments were submitted for sequencing to the DBS Automated Sequencing Facility at UC Davis. Sequences were determined with ABI PRISM dye terminator cycle sequencing with dRhodamine terminator chemistry. The reactions were electrophoresed on an ABI PRISM 377 DNA sequencer (Applied Biosystems) using a 5% Long Ranger gel. The data were analyzed using ABI Prism Á Sequencing 2.1.1 software (Applied Biosystems).

Primer design. The GenBank database was searched for sequences with high similarity to the DNA fragments. As there were no *Peronospora* sequences in the database at that time, the closest matches were ITS sequences from eleven species of *Phytophthora* (2). These were aligned with the *Peronospora sparsa* sequence using the software of the Genetics Computer Group, University of Wisconsin, Madison. A number of regions that were unique to the *Peronospora* sequence were identified. Unique sequences near the beginning of the ITS1 were used to design several forward primers. Other unique sequences, near the end of the ITS2, were used to design reverse

primers. Candidate primer sequences were entered into the program Primer Designer (ver 2.0; Scientific and Educational Software, Durham, NC) to evaluate the potential for problems due to formation of hairpin loops or primer dimers. Primers were synthesized by the Molecular Structure Facility at UC Davis. Various primer pairs were evaluated for specificity by including them in reaction mixtures with DNA from other fungal pathogens of rose, including *Phytophthora cactorum*, *Botrytis cinerea*, and a *Phragmidium* sp. These fungi were all isolated from rose, and mycelium or spores were sonicated in PCR buffer as described above.

Preparation of rose DNA. Preliminary results indicated that DNA extracted from rose tissues using standard protocols for plants (4,15) was not suitable for amplification, presumably due to inhibition of Taq polymerase by plant compounds. Successful results were obtained with either a phenol-chloroform extraction protocol optimized for another rosaceous plant (17) or with a modification of the manufacturer's protocol for the DNeasy Plant Mini Kit (Qiagen, Inc.). The DNeasy kit was selected for use in all tissue-assay extractions due to its ease of use and quick processing time. The procedure is based on the selective binding of the DNA to a silica gel membrane in the presence of high concentrations of chaotropic salt and then elution of the DNA off of the membrane by a low-salt buffer. Fresh rose tissue (100 to 200 mg) was ground to a powder in a mortar and pestle with liquid nitrogen. These powdered samples were stored in cryogenic vials at -80°C until further processing. The major modifications to the manufacturer's extraction protocol were the replacement of the supplied lysis buffer and elution buffer. For the lysis step, the buffer contained 2.5% cetyltrimethylammonium bromide (CTAB), 1% polyvinyl pyrrolidone (PVP-40), 1.4 M NaCl, 50 mM EDTA, and 100 mM Tris-HCl (pH 8.0). Just prior to use, 0.5% 2-mercaptoethanol was added to the CTAB lysis buffer. For the elution step, the elution buffer provided was replaced with 10 mM Tris-HCl at pH 9.0. The elution step was conducted twice with 100 μl of buffer each time for a final elution volume of 200 μl .

Reactions including the specific primers were carried out as described above for ITS-PCR. To determine the number and size of amplified DNA fragments, 10 μl of each reaction was examined by electrophoresis in 1.5% agarose gels in 0.5% Tris-borate EDTA buffer. Gels were stained with ethidium bromide and DNA was visualized with UV light. Any samples that tested negative with the specific primers were retested with the conserved primers to determine if the DNA was suitable for amplification.

PCR reactions sometimes were inhibited by rose tissue; therefore, optimization of

the protocol was conducted with various concentrations (0.1, 0.2, 0.3, 0.4, and 0.5%) of nonfat dry milk (Difco Laboratories, Detroit) added to the reaction mixture to neutralize inhibitory compounds (3). The sensitivity of the PCR assay was evaluated by spiking DNA extracted from healthy rose cortex tissue (4 ng of DNA per reaction) with various dilutions of *P. sparsa* DNA (ranging from 0.4 to 200 pg of DNA per reaction). For this purpose, concentrated suspensions of *P. sparsa* sporangia were processed with the DNeasy Plant Mini Kit (Qiagen Inc.). The DNA was quantified visually by staining with ethidium bromide and comparing the intensity with DNA standards of known concentration.

PCR assay of field samples. In January and February 1999, rootstock cuttings of cvs. Manetti and Dr. Huey were selected randomly from eight recently planted commercial fields. Fifteen cuttings per field were selected and the emerging vegetative buds were removed and processed separately from the cane. Each cane was processed through an electric pencil sharpener which was cleaned between samples. For each cane, the resulting fragments were mixed and two 200-mg samples each were ground further in liquid nitrogen for extraction of DNA as described above. DNA also was extracted from buds (typically two per cutting) ground in liquid nitrogen. Therefore, for each cutting, three assays were performed; one on DNA from buds and two on DNA from the cane.

In December 1999, samples of crown tissue were collected from 20 mother plants. These plants were selected due to their location in a section of a block that had severe foliar downy mildew symptoms in the spring of 1998. Cortex discs (collected with a 1-cm cork borer) from two plants were combined and chilled overnight on ice. These 10 samples subsequently were ground in liquid nitrogen and DNA was extracted as above. Samples from 16 additional crowns were obtained in February 2000 and processed individually.

Root samples also were collected from miniature roses from a commercial nursery that had a downy mildew outbreak. From each of four plants sampled, 200 mg of crown tissue and two 200-mg samples of root tissue were collected and processed as described above.

Preparation of samples for microscopy. Rose stem tissues were collected from symptomatic field-grown plants and inoculated greenhouse plants and stored in a solution of formaldehyde-alcohol-acetic acid immediately after collection (8). Tissue was prepared for embedding with paraffin using a Leica TP 1020 Automatic Tissue Processor (Leica, Deerfield, IL). The material was dehydrated in a series of ethanol solutions at 10% increments from 50 to 80% for 1 h each. Samples then were

transferred to 1% Eosin in 95% ethanol for 1 h followed by 2 h in 100% ethanol. The ethanol series was followed by 2 h in Hemo-De (Fisher Scientific Co., Pittsburgh, PA), 4 h in Hemo-De with paraffin chips, and 11 h in melted paraffin at 60°C. All steps were performed under vacuum with vertical agitation to assist infiltration. The material was embedded in paraffin blocks using a Leica EG1160 Histo Embedder, sectioned with a Microm HM340E rotary microtome (Carl Zeiss, Inc., Thornwood, NY) at 10 and 12 µm, and placed onto glass slides by floating them in a 42°C water bath. The slides then were placed on a slide warmer at 45°C for 24 h. The staining procedure was initiated by removing the paraffin by immersion in two washes of 100% Hemo-De and one wash of 50% Hemo-De in ethanol. Sections then were hydrated in a four-step ethanol series from 100 to 50% ethanol. Hydrated sections then were stained in 1% Aniline Blue (C.I. 42755) in 0.067 M K₂HPO₄ (7). Sections were either mounted in water and examined immediately or dehydrated through an ethanol series and mounted in Permount (Fisher Scientific). Specimens were examined with a Microphot-SA microscope equipped for epifluorescence microscopy and differential interference contrast microscopy (Nikon Inc., Melville, NY). Colonization of tissues was diagnosed by the observation of oospores or the characteristic haustoria (6,25).

Evaluation of preplant treatments of propagation materials. Trials were conducted to evaluate fungicidal or hot-water dips of dormant cuttings for the control of downy mildew. Four trials were conducted in commercial rose nurseries in the vicinity of Wasco, CA and, in 1999 to 2000, one trial was replicated on the UC Davis campus. In commercial fields, normal grower

practices were used with the exception that standard fungicide sprays were not applied. All trials were conducted with the rootstock cv. Manetti's Rose due to its high susceptibility to downy mildew. The trial fields were established with cuttings that had been treated with bleach (5 min in 0.5% sodium hypochlorite) and rooting hormone (0.3% indole 3-butyric acid), which are standard industry practices. Hardwood rootstock cuttings measured about 30 cm in length with all but two buds per cutting removed. These cuttings subsequently were stored at 4°C for up to 4 weeks before planting in November. The dormant cuttings were treated immediately prior to planting by submersion in fungicide solutions for 10 min. Hot-water treatments varied in temperature and duration (Tables 1 and 2). Spacing between the planted cuttings was 15 cm within the row and 103 cm between rows (except in 1997, when the cuttings were planted in double rows with 103 cm between bed centers). The soil in the Wasco area was a sandy loam, while the soil in Davis was a fine-silty loam. An experimental unit (plot) was a single bed 6.1 m long. Nontreated buffer rows were included between treated rows. Trials were conducted five times over 3 years, although not all treatments were included in all trials. Except where noted, all trials contained five replications arranged in a randomized complete block design.

The following fungicides were used in these studies: azoxystrobin (Quadris 2.08SC, 249.3 g a.i. liter⁻¹; Syngenta Crop Protection, Greensboro, NC), dimethomorph (Acrobat 50WP, 50% a.i.; BASF, Research Triangle Park, NC), fosetyl-Al (Aliette 80WDG, 80% a.i.; Aventis Crop-Science, Research Triangle Park, NC), mefenoxam (Ridomil Gold WP, 44% a.i.;

Syngenta), and metalaxyl (Ridomil 2E, 239.7 g a.i. liter⁻¹, Syngenta). Rates are provided in Tables 1 and 2.

In all trials, disease assessments were made two times per week through the spring until all plants were infected or until symptoms stopped appearing. Disease incidence was defined as the proportion of plants with downy mildew symptoms. From these measurements, the area under the disease progress curve (AUDPC) was calculated as described by Shaner and Finney (18). The AUDPC calculations were based on disease incidence over a 75-day period in 1998 (27 January to 10 April) and a 29-day period in 2000 (6 March to 3 April). In 1999 and 2000, a subset of the plants within each plot was harvested at budding and oven dried for weight measurements.

Statistical analyses. Analysis of variance (ANOVA) was used to evaluate differences in disease incidence, dry weight, and shoot length between treatments. Analyses were conducted with the general linear models (GLM) procedure of SAS (version 6.12; SAS Institute Inc., Cary, NC). Treatment means were compared by Fisher's least significant difference (LSD) test at $P = 0.05$. Due to differences in treatments and experimental design between trials, data from each trial were analyzed separately.

RESULTS

PCR with conserved primers. The primers ITS4 and ITS5 directed amplification of a roughly 820-bp fragment from DNA extracted from sporangia of *P. sparsa*. Two products were detected from DNA from symptomatic rose tissues, one the same size as that from sporangial DNA, and a second, smaller product. This smaller fragment was the same size as that produced by amplification of DNA extracted from healthy rose tissue, and is presumably due to amplification of rose

Table 1. Effect of preplant fungicidal and hot-water dips on incidence of downy mildew in rose nurseries, Kern County, CA

Treatment ^a	AUDPC ^b		
	1998 trial	2000 trial 1	2000 trial 2
Control	2,217.2 a	111.5 a	48.1 a
Azoxystrobin (10,000 mg a.i./liter)	2,038.3 a	...	...
Azoxystrobin (1,000 mg a.i./liter)	...	45.4 c	...
Dimethomorph (1,000 mg a.i./liter)	...	55.4 bc	...
Fosetyl-Al (10,000 mg a.i./liter)	1,987.9 a	55.7 bc	...
Hot water (44°C for 15 min)	...	44.2 c	...
Hot water (46°C for 10 min)	...	107.9 ab	...
Mefenoxam (100 mg a.i./liter)	...	36.5 c	...
Mefenoxam (500 mg a.i./liter)	...	40.0 c	17.7 b
Mefenoxam (1,000 mg a.i./liter)	...	26.3 c	...
Metalaxyl (10,000 mg a.i./liter)	783.0 b	...	...
Metalaxyl (5,000 mg a.i./liter)	662.6 b	...	...
LSD, $P = 0.05$	501.0	54.6	24.5

^a Area under the disease progress curve calculated from twice-weekly assessments of the percentage of plants with downy mildew symptoms during the periods 27 January to 10 April 1998, and 6 March to 3 April 2000. Means in the same column followed by a common letter are not statistically different ($P = 0.05$) according to Fisher's least significant difference (LSD) test. Values are means of four replications (1998 trial) or five replications (2000 trials).

^b Cuttings of rootstock 'Manetti' were submerged for 10 min (unless otherwise noted) immediately prior to planting.

Table 2. Effect of preplant fungicidal or hot-water dips on rose shoot dry weight at four months after bud break, 1999

Treatment ^a	Rate (mg a.i./liter)	Dry weight ^b (g)
Control	0	164.0 a
Fosetyl-Al	10,000	164.0 a
Azoxystrobin	1,000	148.0 ab
Metalaxyl	100	136.0 ab
Dimethomorph	1,000	128.0 bc
Metalaxyl	1,000	128.0 bc
Metalaxyl	10,000	104.0 cd
Hot water ^c	...	92.0 d
LSD, $P = 0.05$	...	30.1

^a Cuttings of rootstock 'Manetti' were submerged for 10 min (unless otherwise noted) immediately prior to planting.

^b Means followed by a common letter are not statistically different ($P = 0.05$) according to Fisher's least significant difference (LSD) test. Values are means of five replications.

^c Temperature of 46 to 47°C for 15 min.

rDNA. Primers ITS4 and ITS5 also directed amplification of a roughly 820-bp fragment from DNA of *Phytophthora cactorum*. In reactions containing DNA of *Botrytis cinerea* and *Phragmidium* sp., the amplified fragments were smaller and larger, respectively, than those amplified from the oomycetes *Peronospora* and *Phytophthora*. Reliable amplification of DNA extracted from rose tissue required the inclusion of 0.4% nonfat milk in the PCR mix.

PCR with specific primers. From the ITS sequences of four isolates of *P. sparsa*, a forward primer (PS3, 5' ATTTGTGCTGGCTGGC 3') and a reverse primer (PS1, 5' TGCCACACGACCGAAGC 3') were designed. These primers reproducibly directed amplification of a 660-bp fragment from extracts from sporangia of all 15 tested isolates of *P. sparsa*. These isolates included 10 from field-grown roses in Kern County; 1 from a rose greenhouse in Contra Costa County, CA; 3 from wholesale ornamental nurseries in Solano County, CA, and in Oregon; and 1 from commercial caneberrys in Santa Cruz County. These primers also directed amplification of the same size fragment from 100% of DNA extracts from symptomatic rose leaf and stem cortex samples ($n = 64$). No PCR products were detected from reactions containing DNA extracted from healthy rose tissue ($n = 11$) or from reactions with DNA from other rose pathogens, including *Phytophthora cactorum*, *B. cinerea*, and *Phragmidium* sp. (two or three isolates of each species). In reactions containing 4 ng of DNA extracted from healthy rose cortex, the detection limit was 2 pg of *P. sparsa* DNA.

PCR assay of field samples. Positive PCR results never were obtained from DNA extracted from rootstock cuttings collected from recently established commercial production fields ($n = 92$ cuttings). However, the *Peronospora*-specific PCR

product was detected in 17% of the samples ($n = 26$) of crown of asymptomatic rootstock mother plants which exhibited foliar symptoms the previous season. In addition, 73% of root samples from symptomatic miniature rose plants were positive ($n = 22$) for *P. sparsa* and all four crowns tested positive.

The specific primers always directed amplification of the 660-bp *Peronospora* fragment from DNA extracts of stem epidermal and cortex tissues from inoculated, symptomatic canes ($n = 14$), but produced PCR products less consistently from extracts of pith plus vascular tissues (5 out of 15).

Microscopy. The aniline-blue staining method allowed clear differentiation of pathogen structures from plant tissue when viewed with epifluorescent microscopy (Fig. 1). Mycelia and diagnostic haustoria were always visible within symptomatic canes. The mycelium was located intercellularly between epidermal and cortex parenchyma cells. The pathogen never was observed by microscopy within vegetative buds collected from rootstock cuttings ($n = 27$ buds).

Oospores formed profusely in the mesophyll of symptomatic leaves and the cortex parenchyma of stems from both field and artificially inoculated plants (Fig. 1). Interestingly, abundant oospores formed in leaves inoculated with either of two single-lesion isolates, suggesting that *P. sparsa* may be homothallic (B. Aegerter and R. Davis, unpublished).

Attempts to examine crown and root tissues by microscopy were not successful due to the failure of the paraffin to fully penetrate these lignified tissues.

1998 field trial. Bud break occurred in January and downy mildew appeared immediately thereafter and was severe in many fields in Kern County through mid-May. There was a positive correlation between the variances and the means of the AUDPC of the treatments; thus, the data

were log transformed prior to analysis. However, the results of the statistical tests were comparable between transformed and nontransformed data; therefore, the non-transformed data and analysis are presented (Table 1). There were significant differences in the AUDPC between treatments ($P < 0.0001$). Compared with the nontreated control, metalaxyl applied at 5,000 and 10,000 mg a.i./liter reduced the AUDPC by 70 and 65%, respectively, whereas azoxystrobin and fosetyl-AI did not significantly affect disease incidence (Table 1). No phytotoxicity symptoms or growth reductions were observed.

1999 field trials. Downy mildew did not occur in any commercial rose fields in Kern County during the spring of 1999; therefore, disease severity data were not collected. However, 4 months after bud break, a subset of plants from each plot was harvested to evaluate the visually observable variation in growth between plots. Subsequent analysis of dry weight measurements indicated that this variation was attributable to a treatment effect ($P = 0.0002$). The hot-water dip, dimethomorph, and the two higher rates of metalaxyl (1,000 and 10,000 mg a.i./liter) reduced dry weight over the control treatment (Table 2). The hot-water dip also reduced the stand establishment by 65% compared with the control plots ($P = 0.0001$), whereas no other treatments had a significant effect on stand (*data not presented*). Azoxystrobin, fosetyl-AI, and metalaxyl at 100 mg a.i./liter had no effect on dry weight (Table 2). Shoot length at 2 months after bud break also varied between treatments ($P = 0.01$); fosetyl-AI significantly increased shoot length, whereas the other treatments had no effect (*data not presented*).

2000 field trials. In Kern County, downy mildew did not occur at bud break in February but did appear from the second week of March through the first week of April. There were significant differences in AUDPC between treatments ($P = 0.025$). Mefenoxam (the active enantiomer contained in metalaxyl), hot water (44°C for 15 min), azoxystrobin, dimethomorph, and fosetyl-AI reduced disease incidence relative to the control (Table 1). The shorter hot-water dip (46°C for 10 min) had no effect on disease (Table 1). Shoot weights were not significantly different between treatments ($P = 0.258$), but trends were similar to what was observed in 1999 (i.e., fosetyl-AI resulted in the most growth and mefenoxam at 1,000 mg a.i./liter resulted in the least growth; *data not presented*). In a replication of this trial conducted at UC Davis during the same time period, no disease occurred and there were no differences in dry weights between treatments ($P = 0.93$, *data not presented*). In a third trial conducted in Wasco, which included a variety of control measures, a preplant dip with mefenoxam at 500 mg a.i./liter reduced disease by 63% ($P = 0.026$, Table 1).

Fig. 1. *Peronospora sparsa* within rose stem cortex tissue; haustorium (left) viewed with epifluorescent microscopy ($\times 600$ magnification) and oospores (right) viewed with differential interference contrast microscopy ($\times 200$ magnification).

DISCUSSION

The PCR-based technique developed here for the detection of *P. sparsa* has commercial application in the detection of the pathogen in propagation materials or harvested plants. In this study, the technique was used to reveal the pathogen within rose stems, crowns, and roots, including the crowns of symptomless rootstock mother plants that were highly lignified. A major challenge in the development of a reliable PCR assay for roses was the inhibition of the reaction by plant compounds. This was presumably due to inhibitors of Taq polymerase activity such as tannins, polyphenols, and polysaccharides that tend to cause problems of this nature when working with plants in the family *Rosaceae* (17). In our experiments, reliable DNA amplification was obtained through use of a silica-gel membrane extraction method with a CTAB extraction buffer containing high salt concentrations and PVP. Finally, addition of nonfat milk to the reaction mixture improved amplification. The mechanism by which the milk attenuates the inhibition is not known (3).

The results of the PCR assay and microscopic observations confirm previous reports that *P. sparsa* colonizes stem cortex tissue (14). However, this is the first report of *P. sparsa* occurring in crown and root tissues of rose. The less consistent occurrence of *P. sparsa* in vascular tissues suggests that the pathogen must be colonizing the crown by mycelial growth through the stem cortex.

The role of oospores in the disease cycle of rose downy mildew is unknown. Baker (1) observed oospores in rose leaves but found their occurrence only sporadic. Although we found numerous oospores in both leaves and stems, epidemiological importance does not necessarily follow. Mycelium is formed abundantly in the stem and the host is perennial and vegetatively propagated; therefore, the pathogen likely need not rely upon survival of its oospores. As a sexual spore, they might be the product of out-crossing and hence a source of genetic variation. However, preliminary results from inoculations with two single-lesion isolates suggest that *P. sparsa* may be homothallic.

Perennating crown infections previously have been demonstrated in downy mildews of other crops. The hop downy mildew pathogen, *Pseudoperonospora humuli*, overwinters as mycelium in the perennial crown of hop plants (19). Crowns become infected by mycelial growth of the pathogen downward from systemically infected spikes or by zoospores that infect stem bases (19). Such infections have been demonstrated to be a source of inoculum for epidemics the following season (9). In onion, mycelium of *Peronospora destructor* is reported to survive within bulbs (16). Using microscopy, Tate (20) demonstrated what he called "systemic" cortex infection

of boysenberry (*Rubus loganobaccus*) by *P. sparsa*. In this host, the pathogen seems to overwinter in the crown tissue and grows along with the emerging canes the following season. Tate (20) suggested that the practice of taking cuttings from production blocks ensured disease transmission into new plants and production areas. In arctic bramble (*R. arcticus*) cultivated in Finland, downy mildew caused by *P. sparsa* was observed on new growth from roots collected from commercial fields and planted in a growth chamber (12). In roses, it is possible that transmission of the pathogen to new growth from infected crowns or canes may likewise occur.

The use of systemic fungicides to decrease survival of downy mildews in perennial, woody plant parts previously has been demonstrated in hops (9) and caneberries (21). In hops, controlling downy mildew with metalaxyl in one year reduced the number of systemically infected shoots (primary infections) the following spring, while spraying metalaxyl over new shoots emerging from systemically infected crowns reduced secondary infections (9). In caneberries, metalaxyl soil drenches conducted at transplanting reduced incidence of downy mildew on suckers arising from infected crowns (21). Preplant dips of infected crowns in metalaxyl were not effective in this system (21).

In our studies, preplant dips of dormant rose cuttings in metalaxyl effectively reduced incidence of downy mildew on shoots emerging from these cuttings. Although the higher concentrations of metalaxyl (1,000 mg a.i./liter or higher) reduced subsequent growth from the cuttings, lower concentrations (100 and 500 mg a.i./liter mefenoxam) were not phytotoxic and effectively controlled downy mildew. Metalaxyl and mefenoxam are closely related; the former is a racemic mixture of active and inactive enantiomers while the later contains only the active enantiomer. Fosetyl-Al was not effective under high disease pressure but was effective under moderately disease-conducive conditions; further testing is needed. The efficacy of dimethomorph, azoxystrobin, and hot-water dips should be evaluated in future trials. A hot-water dip, which could avoid the pesticide registration process, would be particularly useful if the temperature and duration could be optimized to maximize downy mildew control and minimize damage to the plant. In studies on the control of nematodes with hot-water treatment, damage to rose roots was reduced by prior storage of the dormant plants at 38°C for 24 h to induce heat-hardening (22).

Success in controlling downy mildew by dipping rose cuttings in fungicides or hot water supports our conclusion that *P. sparsa* overwinters in woody tissue. Preplant dip treatments may delay the start of a mildew outbreak or reduce its severity,

presumably by reducing the number of perennating infections serving as initial inoculum (23). A more economical solution, in the long term, may be to reduce incidence of the pathogen in propagation materials by chemically treating or removing infected rootstock mother plants. Plants could be tested by PCR and eliminated if positive. This approach, which has worked well in reducing the transmission of viruses in roses, would be successful if secondary spread of the pathogen was limited.

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