Hyaloperonospora camelinae on *Camelina sativa* in Washington State: Detection, Seed Transmission, and Chemical Control

E. M. Babiker and S. H. Hulbert, Department of Plant Pathology, Washington State University, Pullman 99164-6430; and T. C. Paulitz, United States Department of Agriculture–Agricultural Research Service, Root Disease and Biological Control Research Unit, Pullman, WA 99164-6430

Abstract

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Camelina (*Camelina sativa*) plants with symptoms of downy mildew were obtained from three different locations in Washington State. Based on polymerase chain reaction (PCR) and sequencing of the internal transcribed spacer (ITS)1-5.8S-ITS2 region, the causal pathogen was identified as *Hyaloperonospora camelinae*. The PCR primers consistently amplified 699-bp bands from the infected plants but not from the asymptomatic plants. A comparison of the sequences with those in GenBank revealed 100% sequence similarity to *H. camelinae*. Growth and development of the *H. camelinae* was observed in different tissues using light microscopy and scanning electron microscopy

(SEM). Light microscopic observation revealed the presence of oospores in the infected leaves and SEM revealed the presence of conidia and conidiophores on the seed surface. To determine whether *H. camelinae* is a seed-transmitted pathogen, seed collected from infected plants were planted in Sunshine professional growing mix maintained in a growth chamber. Disease symptoms were observed in 96% of the seedlings compared with 3% of the seedlings grown from seed from asymptomatic plants, which indicates that *H. camelinae* is a seed-transmitted pathogen. Seed treated with mefenoxam, a fungicide specific for Oomycetes, significantly reduced the incidence of the disease.

Camelina (*Camelina sativa* (L.) Crantz) is an oilseed crop in the mustard family that has been cultivated in Europe since antiquity (19). It was more widely cultivated in Europe before World War II, and was introduced into North America as a weed in the 18th century, going by the name of false flax or gold-of-pleasure (7). Several characteristics have resulted in a renewed interest in camelina as an oilseed crop: its ability to grow in a dry climate (11), low nutrient requirements, and oil high in omega-3 fatty acids (9), which have health benefits (14). Recent interest in the crop as a biofuel feedstock has also stimulated research for both biodiesel and aviation fuel (26). It was investigated as an oilseed crop in Canada in the 1960s and in Minnesota in the 1980s (21). Within the last 5 years, this crop has been grown on limited commercial acreage in the low-rainfall regions of Montana and the inland Pacific Northwest (PNW) (16,17).

Camelina can be attacked by a number of diseases and pests, including downy mildew (25). *Peronospora camelinae* was described by Gäumann in 1918, from a previously described taxon, *P. parasitica* var. *camelinae*, described by Kühn in 1864 (23). There were reports of both *P. parasitica* and *P. camelinae* on camelina in Europe in the mid-20th century (3,5), and both have been reported from Poland, Austria, Switzerland, Spain, Portugal, Bulgaria, and Germany (6). The first report of downy mildew on camelina in N. America was from Canada in 1955 (2). Prior to the 2000s, there was no report of this disease from the United States in plant pathology literature but it was recently reported from Oregon and the high plains of the United States in 2009 and 2011 (10,20). Both *P. camelinae* and *P. parasitica* were recently moved to the genus *Hyaloperonospora* (4).

Since spring 2010, there has been an increase in incidence and severity of downy mildew disease in different locations in Wash-

ington State. Disease symptoms appeared as white mycelial growth on stems and developing pods, twisted stems, poor flowering, and empty pods (25). Chlorotic lesions were occasionally seen on leaves. The distinction of *Hyaloperonospora* spp. based on morphological characteristics is laborious, time consuming, and likely less reliable than molecular analyses. Therefore, an improved method of detection using a polymerase chain reaction (PCR)-based assay is needed because both *Hyaloperonospora camelinae* and *H. parasitica* are obligate parasites that grow only on living hosts. PCR approaches have been successfully used to detect the presence of fungal and Oomycete pathogens in plant tissues (18,22). Recently, the sequences of the complete ITS1-5.8S-ITS2 region of *H. camelinae* and *H. parasitica* have become available (8). This sequence can be used to design species-specific primers to detect the presence of either of *H. camelinae* and *H. parasitica* in plant tissues.

The incidence of downy mildew in fields with no previous history of camelina raises the question of whether *H. camelinae* is seed transmitted or not. Transmission of *H. camelinae* through seed has not been documented. Infected seed could be a main source of inoculum, especially in areas where the pathogen does not exist, and, thereby, extend the geographical distribution of the disease. To develop effective management strategies for downy mildew, it is important to know whether *H. camelinae* is seed transmitted or not, and how effective a seed treatment would be. Little research has been done on seed treatments for this crop. Mefenoxam seed treatment has been found to be effective in reducing the incidence of downy mildew in sunflower and pearl millet (22,27). The efficacy of mefenoxam for controlling *H. camelinae* has not been investigated. Therefore the objectives of this study were to (i) identify the downy mildew pathogen of camelina in Washington State, (ii) develop a reliable PCR-based assay to detect the presence of the pathogen in the seed, (iii) determine whether *H. camelinae* is a seed transmitted pathogen, and (iv) test the efficacy of mefenoxam as a seed treatment for controlling downy mildew of camelina.

Materials and Methods

Design of *H. camelinae*- and *H. parasitica*-specific primers. The sequences of the ITS1-5.8S-ITS2 region of *H. camelinae*

Corresponding author: S. H. Hulbert, E-mail: scot_hulbert@wsu.edu

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(EU049217) and *H. parasitica* (EU049279) were obtained from GenBank (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). The ITS sequences from the two species were aligned using CLUSTAL W in BioEdit 4.0.6.2 (<http://www.mbio.ncsu.edu/BioEdit/page2.html>) and three specific PCR primers were designed from nonconserved regions to amplify 802 and 584 bp from *H. parasitica* and 699 bp from *H. camelinae*. (Table 1).

PCR assays using *H. camelinae*-specific primers. Samples of diseased camelina plants were collected from three different locations in Washington State, near Lind, Dusty, and Pullman. The genomic DNA was extracted from the fungus mycelium that was carefully removed from the infected plants using a sterile blade, and about 0.2 g of the collected mycelia were placed in 1.5-ml Eppendorf tubes and ground in liquid nitrogen. Following grinding, 500 µl of warm DNA extraction buffer (100 mM Tris-HCl, 20 mM EDTA, 2 M NaCl, 2% cetyltrimethylammonium bromide, and 0.3% NaHSO₃ [pH 8.0]) was added and mixed. The tubes were incubated in a water bath at 65°C for 45 min, with hand agitation every 5 min. After incubation, 500 µl of chloroform: isoamyl alcohol (24:1, vol/vol) was added to the tubes and the contents were mixed and centrifuged at 10,000 × g for 5 min. The supernatant was transferred to a new tube and an equal volume of isopropyl alcohol was added. The tubes were inverted gently, incubated for 5 min at –20°C, and centrifuged at 10,000 × g for 5 min at 4°C. After discarding the supernatant, the DNA pellet was washed in 500 µl (70%) of ethanol, air dried, and resuspended in 100 µl of water. For negative control, seed from asymptomatic plants were collected and placed in a beaker with 0.5% sodium hypochlorite (NaOCl) and stirred with a magnetic stir bar for 5 min. Seed were rinsed in water and planted in Sunshine professional growing mix, and 2-week-old leaves were used for DNA extraction. The PCR reaction was carried out in 20-µl volumes using 100 ng of genomic DNA, 1× buffer, 1.5 Mm MgCl₂, 0.2 mM each dNTP, 0.5 µM each primer pair, and 1 unit of Taq polymerase. The PCR reactions were performed following 5 min of incubation at 94°C with 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. A final extension of 7 min at 72°C was performed. PCR products were separated by electrophoresis in 1.5% (wt/vol) agarose gels stained with ethidium bromide. The desired DNA fragments were recovered and purified by a QIAquick gel extraction kit (Qiagen, Inc.) following the manufacturer's protocol. The purified fragments were ligated to the pGEM-T easy vector system kit (Promega Corp.) and 4 µl of the ligation mixture was transformed to 100 µl of *Escherichia coli* strain TOP10. Based on the PCR results using HC and HP primer pairs, positive clones with the desired fragment were grown overnight on 2 ml of liquid Luria-Bertani broth supplemented with ampicillin at 100 µg ml^{–1}. Plasmid DNA was extracted using a Pure Yield plasmid miniprep kit (Promega Corp.). Recombinant clones were sent to Elim Biopharmaceutical Inc. for sequencing and a BLAST search was used to compare the obtained sequences with sequences in the GenBank database.

Detection and transmission of *H. camelinae*. To investigate whether infected seed can give rise to infected camelina plants, seed from infected camelina plants were planted in tapered plastic cones (4 cm in diameter by 20 cm long; Ray Leach Cone-tainer) filled with Sunshine professional growing mix (SunGro). Cones were maintained in a growth chamber programmed for 12°C with a 12-h photoperiod and watered uniformly at 4-day intervals. High humidity was maintained by partially covering the 2-week-old seedlings with plastic for 6 weeks. At the flowering growth stage, plants were inspected for visual symptoms of downy mildew and the percentage of infected plants was calculated for each replicate. Leaf samples were collected from infected and asymptomatic plants for DNA extraction. The experiment was repeated twice and the data from repeated experiments were tested for equality of variance.

Effect of mefenoxam seed treatment on incidence of downy mildew. Seed from infected plants were treated with mefenoxam or rinsed with 0.5% NaOCl, stirred with a magnetic stir bar for 5

min, and then treated with mefenoxam. Mefenoxam (Apron XL at 3 g a.i./kg of seed; Syngenta Crop Protection, Inc.) was diluted in a volume of double-distilled water equivalent to 15% of seed weight. Seed were placed with the fungicide in a plastic bag and shaken for 1 min. Treated seed were planted in cones filled with Sunshine professional growing mix. Control cones were planted using non-treated seed collected from the same infected plants. Seedlings were maintained in a growth chamber programmed as previously described. The experiment was repeated twice. Plants were inspected for visual symptoms of downy mildew at the flowering stage and the percentage of infected plants was calculated for each treatment.

Inoculation of canola with *H. camelinae*. The pathogenicity of *H. camelinae* on canola was assessed in a growth chamber experiment. The conidial suspension was prepared by immersing infected leaves and stems in water for 24 h with shaking. The conidial concentration was quantified with a hemacytometer and adjusted to 5 × 10⁴ spores/ml. Healthy canola seedlings of 'Virginia' and 'Rosini' were inoculated by pipetting 500 µl of conidial suspension of *H. camelinae* on the upper surface of the lower leaf. Asymptomatic camelina seedlings of 'Cheyenne' were inoculated and maintained in the same growth chamber as the control. In other experiments, seed of two canola cultivars, Virginia and 'Wichita', were germinated in two petri dishes with filter paper saturated with 2 ml of conidial suspension of *H. camelinae*. The petri dishes were incubated in a growth chamber programmed for 12°C with a 12-h photoperiod for 3 days and the germinated seed were then planted in plastic containers filled with Sunshine professional growing mix. The infected plants were maintained in a growth chamber at 15°C and loosely covered with plastic bags for 6 weeks. Plants were monitored visually for symptom development on leaves and microscopically examined for the growth of the pathogen.

Light and scanning electron microscopy. To verify the presence of conidiophores, conidia, and oospores in camelina leaves and seed coats, dry leaves and seed were soaked in water for 3 days with shaking. Small leaf and seed coat segments were floated on glass slides, covered with coverslips, pressed gently, and examined with a bright-field light microscope at ×40.

Scanning electron microscopy (SEM) was used to examine the internal tissue of the infected camelina stems. Stems pieces were collected from infected seedlings and placed in a fixative solution consisting of 2.5% glutaraldehyde and 2% paraformaldehyde buffer. The specimens were then treated with 1% osmium tetroxide for 1 h and dehydrated through a graded ethanol series for 10 min before critical point drying. The tissues were coated with gold and examined using the Hitachi S-570 electron microscope. Seed were collected from infected plants, lyophilized for 24 h, and observed under field-emission SEM (Virtis Inc.).

Statistical analysis. Analysis of variance was calculated using Proc GLM in SAS (version 9.1; SAS Institute). Means were compared using Fisher's protected least significant difference at *P* = 0.05. A two-tailed *F* test for equality of variances was used to test whether data from the trials could be combined and analyzed together.

Table 1. List of primer pairs designed for the amplification of the internal transcribed spacer (ITS)1-5.8S-ITS2 region from *Hyaloperonospora camelinae* and *H. parasitica* with the optimum annealing temperature

Primer	Sequence (5'→3')	Annealing temperature (°C)
<i>H. camelinae</i>		
HC-L	attgggagtcgtgatcttgg	60
HC-R	ctcgacaaccgaagtgtgaca	...
<i>H. parasitica</i>		
HP1-L	gatcttggtgtaactactagtctcg	60
HP1-R	actggcctcacggacacac	...
HP2-L	gaaccattgggctttgtct	60
HP2-R	cttgccctacggacaccatt	...

Results

The HC primer pairs were used to detect the presence of *H. camelinae* in the infected plants. The HC primer pair amplified a 699-bp fragment from DNA extracted from infected plants collected at different locations but no amplification was detected from DNA extracted from asymptomatic plants. Comparison of the 645-bp sequences with those in GenBank revealed that the sequences were 100% identical to *H. camelinae* sequences EU049217, AY531456, and AY531457 and 92% similar to *H. parasitica* sequence EU049279. These results indicated that *H. camelinae* is the main cause of downy mildew in Washington State. The HP1 primer pair designed from the ITS1-5.8S-ITS2 region of *H. parasitica* weakly amplified an approximately 400-bp product from infected camelina plants collected from Lind. Comparison of the sequences with those in GenBank revealed that the sequence showed no similarity to the ITS1-5.8S-ITS2 region of *H. parasitica* and was apparently a nonspecific amplification product. When the HP2 primer pair and the combination of HP2L and HP1R were used they did not amplify any products. To evaluate the specificity of the HC primer pair, batches of infected and asymptomatic seed were separately germinated in petri dishes. The HC primer pair consistently amplified a 700-bp DNA fragment from plants grown from the infected seed only. To evaluate the sensitivity of the HC primer pair, 1, 10, and 100 ng of DNA from infected plant material was used in a 20- μ l volume PCR mix. The HC primer pair amplified a visible product from 10 and 100 ng of DNA in 35 cycles of amplification.

To examine the transmission of the pathogen, seed collected from infected camelina plants were planted in cones and maintained in the growth chamber programmed for 12°C with a 12-h photoperiod. Two trials were conducted in two different growth chambers. According to two-tailed *F* tests, variances from the two trials were not significantly different ($P = 0.25$); therefore, data

from the two experiments were pooled and analyzed together. At the flowering stage, the incidence of downy mildew was statistically significant between plants grown from seed collected from infected camelina plants and plants grown from seed collected from asymptomatic plants ($P < 0.0001$). Approximately 96% of the seedlings (192 total) grown from the infected seed developed downy mildew symptoms. In contrast, only 3% of the seedlings from seed collected from asymptomatic plants in the same field developed symptoms. To confirm the presence of *H. camelinae* in the tissue, the HC designed primer pair was used with DNA extracted from the infected plants. The HC-designed primer pair amplified an approximately 700-bp fragment from each of seven of the infected plants tested and DNA sequencing confirmed the identity of the fragment as *H. camelinae*. Sequencing results revealed that all the amplified fragments were 99 to 100% identical to the ITS1-5.8S-ITS2 region of *H. camelinae*. SEM observation revealed that stems and seed were colonized by white-colored conidiophores and conidia (Fig. 1A and B). Most of the observed conidia were subglobose, hyaline, and measured 5 to 18.1 by 3 to 8.3 μ m (Fig. 1C and D). In addition, light-microscopic observation confirmed the presence of conidiophores, conidia, and oospores in camelina leaves but no oospores were observed on the seed surface (Fig. 1E and F). These results indicated that *H. camelinae* is a seed-transmitted pathogen and care should be taken to reduce the spread of the pathogen.

The effect of seed treatment on incidence of downy mildew was studied. The treatment included nontreated infected seed, seed treated with mefenoxam only, and seed rinsed in 0.5% sodium hypochlorite and then treated with mefenoxam. Significantly lower incidence of downy mildew was observed when seed were treated with mefenoxam at 3 g a.i./kg of seed ($P < 0.0001$). Compared with seed treatment with mefenoxam, rinsing in 0.5% sodium hypochlorite and then treating with mefenoxam significantly reduced the incidence of the disease from 39.8 to 11.8 ($P < 0.0001$).

Fig. 1. Scanning electron micrograph of the A, stem and B, seed surface of camelina colonized by conidiophores of *Hyaloperonospora camelinae*. C and D, Field emission scanning electron micrograph of conidia of *H. camelinae* on the surface of camelina seed. Light micrograph of E, conidiophore and F, oospores of *H. camelinae* in camelina leaf tissue.

PCR assays using the HC-designed primer and sequence analysis confirmed the presence of *H. camelinae* in the infected plants. Artificial inoculation using inoculum prepared from infected plants indicated that *H. camelinae* was able to cause downy mildew on camelina but not in canola.

Discussion

Several previous studies have reported downy mildew on camelina caused by *H. parasitica* and *H. camelinae* (10,20,25). PCR with universal ITS primers was used by Putnam et al. (20) and Harveson et al. (10) for identification but they had to sequence the products for verification. Because the sequences of the ITS region of the ribosomal RNA-encoding genes are quite different between the two species, it was relatively simple to design PCR primers that distinguished between the two species without sequencing. The HC primer pair designed from this region appeared suitable for *H. camelinae* detection because it did not yield PCR products from the asymptomatic plants. The HP primer pair weakly amplified an approximately 400-bp product from infected camelina plants collected from Lind but this appeared to be a nonspecific amplification product. These results indicated that *H. camelinae* is the causal pathogen of downy mildew on camelina in Washington State in recent years. To our knowledge, this is the first report of *H. camelinae* in Washington State. Using dilution series, we demonstrated that the HC primer pair is sufficiently sensitive to detect the presence of *H. camelinae* in plant tissue. The PCR assay was faster and less labor intensive than morphological examination, making it possible to screen a large number of samples in a single day.

Seed transmission of *H. camelinae* on camelina was speculated because there had been no previous reports of downy mildew in Washington State, the fields where the disease was observed had not been previously cropped with camelina, and no downy mildew symptoms were observed on other *Brassica* spp. Seed collected from infected plants were planted in cones filled with Sunshine professional growing mix and placed in isolated controlled-environment growth chambers to ensure the absence of any extraneous source of inoculum. Symptom development in the seedlings indicated that infected seed were the only source of inoculum. This finding was further supported by the observation of the conidia and conidiophores on the seed surface using SEM. In the literature of downy mildew pathogens, there are numerous reports of seed transmission. Landa et al. (13) reported that downy mildew of opium poppy caused by *P. arborescens* is a seed-transmitted disease. *Peronosclerospora sorghi*, the causal of the downy mildew of maize, was previously reported by Adenle and Cardwell (1) to be seed transmitted. Introducing infected seed would allow the pathogen to become established in camelina fields with no previous history of downy mildew and it may create a problem in subsequent camelina crops. Oospores were observed in infected leaves, and these could serve as a long-term survival structure in the soil. However, seed treatment with mefenoxam could be an adequate means of control, especially if seed were rinsed in 0.5% sodium hypochlorite before treatment. To our knowledge this is the first report on evaluating the effectiveness of mefenoxam as a seed treatment to control *H. camelinae* on camelina.

The epidemiology of this disease in Western United States is still unknown, but it may be similar to downy mildew of sunflower caused by *Plasmopara halstedii*. This pathogen is capable of infecting roots from oospores in soil, and was the first downy mildew to be demonstrated as a soilborne pathogen (24). *P. halstedii* can also infect the hypocotyl of emerging seedlings, either from inocula on the seed or oospores in the soil (24). The light-microscopy observation revealed the presence of oospores of *H. camelinae* in leaves of camelina. When leaves decompose in the soil, oospores may be released and infect the subsequent camelina crop. This observation revealed the potential of plant debris serving as an inoculum source if leaves are harvested with seed or decompose in the field. In pearl millet downy mildew, the development of the symptoms on adult plants is due to either systemic mycelium or secondary infection (28). Mature tissue of many hosts become

more resistant to infection, and the role of secondary infection by conidia is variable, depending on the environmental conditions and moisture (29). It is not known how important secondary spread would be in eastern Washington because, by the time conidia are produced, the conditions are warm and dry, and rainfall is rare in the summer.

Little work has been done on camelina downy mildew disease management. Polish researchers found that spraying with copper oxychloride and maneb were effective in reducing the disease (33,34). The low economic value of the crop may preclude fungicide sprays but seed treatments may be economical. Response of downy mildew pathogens to metalaxyl and mefenoxam seed treatments was investigated in previous studies. Mefenoxam in conjunction with resistant genotype was found to be effective in controlling *Peronosclerospora sorghi*, the causal agent of downy mildew of sorghum (12,30). Seed treatment with metalaxyl significantly reduced the incidence and severity of downy mildew of pearl millet compare to non treated seed (32).

Besides chemical control, resistant varieties may provide a sustainable control strategy in combination with seed treatment. In camelina, resistance to downy mildew was found to be effective in reducing the incidence of the disease (15,31).

The results from this study indicated that *H. camelinae* is the causal pathogen of downy mildew on camelina and that the designed PCR primers can be used to detect the presence of *H. camelinae* in infected tissue. In addition, we demonstrated that *H. camelinae* is seed transmitted, meaning that seed can serve as a source of inoculum for future crops and dispersal to new areas. Mefenoxam seed treatment can reduce the incidence of downy mildew. Additional work is needed to determine how long the pathogen survives in or on the seed, whether seed infestation can be eliminated, and whether the oospores in soil and infected tissue could serve as inocula for the development of the disease in following crop.

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