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PCR detection of *Pseudoperonospora humuli* in air samples from hop yards

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Downy mildew of hop, caused by *Pseudoperonospora humuli*, is an important disease in most regions of hop production and is managed largely with regular fungicide applications. A PCR assay specific to *P. humuli* and the related organism *P. cubensis* was developed and used to monitor airborne inoculum in hop yards to initiate fungicide applications. The PCR amplified as little as 1 fg of genomic DNA of *P. humuli*, and yielded an amplicon in 70% of reactions when DNA was extracted from single sporangia. In the presence of 25 mg of soil, an amplicon was amplified in 90% of reactions when DNA was extracted from 10 or more sporangia. During nine location-years of validation, PCR detection of the pathogen in air samples occurred no later than 8 days after the appearance of trace levels of disease signs and/or detection of airborne spores in a volumetric spore sampler. Inoculum was detected on average 4·5 days before (range −8 to 14 days) the first appearance of basal spikes in six commercial yards, or 1·3 days after (range −5 to 1 days) sporangia were detected in a volumetric spore sampler in experimental plots. In commercial yards, use of PCR to initiate the first fungicide application led to enhanced disease control or a reduction in fungicide use in four of six yards compared to growers' standard practices. These results indicate that the efficiency and efficacy of hop downy mildew management can be improved when control measures are timed according to first detection of inoculum.

Keywords: aerobiology, copper fungicide, disease management, *Humulus lupulus,* molecular disease detection, *Pseudoperonospora cubensis*

Introduction

Hop (*Humulus lupulus*) is a climbing perennial bine grown for its strobiles (cones), which are used in brewing of beer. The lupulin glands of hop cones produce soft resins (alpha and beta acids) and essential oils that impart flavour and aroma to beer, and also aid in preservation. Nearly all commercial hop production in the USA is located in the Pacific Northwest states of Washington, Oregon and Idaho (Barth *et al*., 1994).

Downy mildew of hop, caused by the oomycete pathogen *Pseudoperonospora humuli*, is an important disease in most regions of hop production in the northern hemisphere (Royle & Kremheller, 1981; Neve, 1991), as well as Argentina (Perez *et al*., 2003). Reductions in cone yield result from infection of bines (i.e. climbing shoots) trained on strings and cone-bearing lateral branches, which arrests shoot development. Later infections of inflorescences and developing cones may cause their abortion, reductions in bittering acids, and quality losses that can render a crop unmarketable (Royle & Kremheller, 1981). In susceptible cultivars, the disease can cause a crown rot that can lead to weak growth during ensuing seasons and in some cases plant death (Skotland, 1961; Johnson *et al*., 1991).

Disease outbreaks are favoured by leaf wetness, high humidity, and temperatures ranging from 8 to 23°C (Royle, 1973; Johnson *et al*., 1983; Johnson & Skotland, 1985), resulting in infection of leaves and shoots that can become systemic and invade the crown and root system (Skotland, 1961; Coley-Smith 1964, 1965). The pathogen perennates as mycelia in systemically infected root systems, invading developing buds during the autumn (Skotland, 1961; Coley-Smith 1964, 1965). The following season, a portion of the infected buds emerge to produce systemically infected shoots (termed 'basal spikes') which serve as a source of primary inoculum to initiate new infections and perpetuate secondary cycles of infection.

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Management of downy mildew relies on regular fungicide applications (Coley-Smith, 1964; Hunger & Horner, 1982), and cultural practices such as spring crown pruning, replanting of diseased plants, and removal of basal spikes (Romanko, 1964; Royle & Kremheller, 1981; Skotland & Johnson, 1983). Various disease forecasting systems have been developed to aid in timing fungicide applications for hop downy mildew in the USA and Europe (Royle, 1973; Johnson *et al*., 1983; Kremheller & Diercks, 1983; Johnson, 1991). A limitation of many of these forecasting systems is the lack of an inoculum availability component or the necessity to enumerate sporangia following air sampling with volumetric spore traps (Royle, 1973; Kremheller & Diercks, 1983). These limitations are likely to prevent more widespread adoption of disease forecasts systems, especially in the USA since hop farms tend to be large (average >250 ha) and deployment of volumetric spore traps is impractical.

Monitoring of airborne inoculum by PCR-based assays has been used to detect and quantify clinical pathogens and allergens (Zeng *et al*., 2006) and monitor airborne phytopathogens (Calderon *et al*., 2002; Fraaije *et al*., 2005; Falacy *et al*., 2007; West *et al*., 2008). Use of PCR-based technologies for detecting inoculum in air samples has not been adopted at the farm-scale, in part because of the expense and inefficiency of the spore traps and technologies used previously. Development of a species-specific PCR assay for *P. humuli* could allow for *in situ* detection of airborne inoculum at a broad scale if inexpensive approaches were available. To this end, research was conducted to develop a PCR and DNA extraction procedure suitable for rapid and sensitive detection of *P. humuli* in air samples. Additionally, the air sampling procedure was validated under field conditions as an aid in determining when fungicide applications should be initiated for management of hop downy mildew.

Materials and methods

Plant material

Plants of the susceptible cultivar Nugget (Haunold *et al*., 1984) were propagated from soft wood cuttings and maintained in a greenhouse isolated by more than 10 km from any hop yards and free of downy mildew. The greenhouse was maintained at approximately 20 to 25°C with a 14 h photoperiod. Plants were grown in Sunshine Mix #1 (SunGro Horticulture) in 1 L pots, watered regularly, and fertilized using Champion 17-17-17 (N-P_2O_5-K_2O) with micronutrients (McConkey's) at each irrigation to promote succulent growth.

Pathogen isolates and inoculum preparation

Basal spikes were collected from commercial hop yards from the major hop growing regions in Oregon, northern Idaho and Washington as described previously (Nelson *et al*., 2004). Sporulation was induced by misting with sterile distilled water before enclosing basal spikes in plastic bags with the stems in a beaker of sterile water and then incubating overnight at room temperature (approximately 20°C) in the dark. Sporangia were harvested by shaking each spike in 15 mL of sterile distilled water and straining the suspension through several layers of cheesecloth. Sporangia were preserved at −20°C in sterile double-distilled water (up to three years) for later DNA extraction (described below).

Monosporangial isolates of *P. humuli* were obtained and maintained on detached leaves of cultivar Nugget as described by Gent *et al.* (2008). Briefly, individual sporangia and a small volume of water were inoculated individually onto the abaxial surface of a leaf collected from the fourth to sixth node. The inoculated leaves were placed onto sterile, moist germination paper in Petri dishes and incubated in a growth chamber at 20°C in a 14 h photoperiod provided by fluorescent lights (approximately 300 μmol m^{-2} s^{-1}). After 3–7 days of incubation, sporulating lesions were apparent on the leaf, and single sporangium isolations were repeated from one of the resulting lesions. Individual isolates were then maintained by weekly bulk transfers of sporangia onto new leaves. When a large quantity of inoculum was needed, individual isolates were increased by removing a mass of sporangia from a purified isolate in approximately 5 μL sterile nanopure water and inoculating several new leaves of cv. Nugget with the sporangia and a small volume of water in approximately 10 locations. Leaves were incubated in sterile Petri dishes.

DNA extraction, sequencing, and primer design

DNA was extracted from sporangia of 59 isolates of *P. humuli* using a MoBio UltraClean Soil DNA kit according to the manufacturer's instructions (MoBio Laboratories) with the following modification. To solution S1, 0·140 g of polyvinyl pyrrolidone (PVP) was added to improve the fidelity of amplification. After the addition of solution S1, 200 μL of inhibitor removal solution (MoBio) was added to the tube with the bead lysis solution. Concentration and quality of DNA extracts were determined fluorometrically (Sambrook *et al*., 1989). DNA was stored in sterile Tris-EDTA (TW) buffer (10 mM Tris and 1 mM EDTA, pH 8·0) or water at −20°C.

A conserved region of the ribosomal DNA internal transcribed spacer region (ITS) was amplified with primers ITS1 and ITS4 (White *et al*., 1990). Primers were synthesized by Integrated DNA Technologies. PCR reactions were carried out in a total volume of 25 μL containing 0·5 μM of each primer, 0·5 μL acetonitrile (50% by volume in water), 10 μL of HotMaster *Taq* DNA Polymerase Mix (5 Prime), 12 μL of PCR-grade water, and 1 μL of template. The amplification program consisted of an initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 51°C for 20 s, and extension at 65°C for 1 min with a final extension at 70°C for 10 min. DNA fragments were electrophoresed in a 1% Tris-acetate EDTA gel. Ethidium

bromide (0·5 μg mL^{-1}) was added to each gel, and the DNA fragments were visualized over a UV transilluminator.

The approximate 923-bp amplicon was gel purified (Qiagen MinElute Gel Extraction Kit, Qiagen) and ligated into pGEM-T Easy Vector (Promega Corporation) and cloned in *E. coli* strain DH5α. The cloned amplicons were sequenced bi-directionally by the Center for Genomics Research and Bioinformatics Core Laboratories (Oregon State University). Sequence alignment was conducted in BioEdit version 7·0 ·9 (Ibis Biosciences). The National Center for Biotechnology Information GenBank non-redundant database was searched for sequence similarity using the BLASTn algorithm (Altschul *et al.*, 1997). After sequence data from several isolates of *P. humuli* was obtained, primers HDM04 (5′AG CCACACAACACATAGT3′) and HDM07 (5′AGA ATTGACTGCGAGTCC3′) were designed from the consensus sequence and used with primers ITS1 and ITS4, respectively, to direct amplification of DNA fragments more specific to *P. humuli*. Amplicons generated by the primers ITS1 and HDM04 were approximately 843 bp and amplicons generated by primers HDM07 and ITS4 were approximately 419 bp. Amplicons were subsequently gel purified and sequenced bi-directionally directly.

A consensus sequence derived from 59 *P. humuli* isolates from the Pacific Northwestern USA and alignment of an additional 23 public sequence data for related species (seven isolates of *P. humuli*, *Pseudoperonospora cannabina, Pseudoperonospora celtidis*, 12 isolates of *Pseudoperonospora cubensis*, *Pseudoperonospora urticae* and *Peronospora destructor*) available in GenBank, indicated that primers HDM04 (forward) and HDM07 (reverse) should be specific to *P. humuli* and *P. cubensis*. The first nucleotide (adenine) of primer HDM07 differed from the sequence data available in GenBank for isolates of *P. cubensis* included in this study. Primer HDM04 was 100% identical to the sequence present in the ITS of all isolates of *P. cubensis*. The amplicon produced by these primers was 338 bp. The amplification program consisted of an initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 58·4°C for 20 s, and extension at 65°C for 1 min with a final extension at 70°C for 10 min. DNA fragments were visualized as described previously.

Specificity and sensitivity assays

The specificity of the PCR assay was verified by performing the PCR with DNA of 45 samples (including hop) representing 35 organisms and isolates (Table 1) as described previously. Additionally, 27 isolates of *P. humuli* and 22 isolates of *P. cubensis* not used in development of the primers and PCR assay were included in the validation to ensure amplification from a diverse collection of isolates. The potential for successful DNA amplification from all isolates was verified in PCR assays with primers ITS1 and ITS4. Results from DNA extractions that did not yield a PCR product with primers ITS1 and ITS4 were not considered valid since amplification was not deemed to be possible. A water blank negative control and *P. humuli* positive control were included in all experiments.

PCR sensitivity was quantified by conducting 10 independent DNA extractions and amplifications on a dilution series of about 0, 1, 5, 10, 100 and 500 sporangia with or without the addition of 25 mg of twice-autoclaved soil (Chehalis silty clay loam) to a silicon grease (Dow Corning) coated glass rod (5 × 1 mm). This soil type is typical of soils used for hop production in Oregon, USA, and was obtained from a fallow field adjacent to an experimental hop yard near Corvallis, Oregon. Additional information on this soil series can be obtained from the USA Natural Resource Conservation Service online database of Official Soil Series Descriptions (http://soils.usda.gov/technical/classification/osd/index.html). The amount of soil added to each extraction corresponds approximately to the maximal amount of soil that can be collected on a Rotorod impaction spore sampler collection rod (SDI Health LLC), and provides a measure of potential PCR inhibition if DNA was extracted from spores collected on a rod under non-sterile field conditions. For the sensitivity analysis, sporangia were collected with the aid of a micropipette and added directly to the reaction tubes for extractions with 1, 5 or 10 sporangia. An aliquot of a dilution series of sporangia was used for the 100 and 500 sporangia densities. DNA extraction and PCR assays were as described above. A nonlinear regression equation was fitted to the percentage of positive PCR reactions for each concentration of sporangia to quantify the PCR sensitivity. Regression analysis was conducted in SigmaPlot version 11·0 (Systat Software).

Field validation of air sampling

Experimental plots

A section of 0·5 ha experimental hop yard planted to cv. Nugget near Corvallis, Oregon (latitude 44·5696, longitude −123·2380) was used for air sampling experiments during 2006 to 2008. The plots were maintained according to standard production practices for hop production in the USA. Removal of hop shoots in early spring and superfluous basal foliage growth is practiced routinely in the USA to remove inoculum of *P. humuli*. To simulate this, early spring growth of plants in the hop yard was removed by a single application of carfentrazone-ethyl (Aim EC, FMC Agricultural Products; 33·6 g a.i. in approximately 467 L of water per hectare) on day of year (DOY) 101, 89 and 107 in 2006, 2007 and 2008, respectively. Superfluous basal foliage was also desiccated using carfentrazone-ethyl, at the rate and application volume indicated above, on DOY 149, 173 and 143 in 2006, 2007 and 2008, respectively.

Disease incidence and severity were assessed regularly (every 3 to 4 days) on all plants in early spring to detect the first signs of downy mildew. After disease was detected, non-treated plots were established consisting of

Table 1 Identity and origin of organisms used to determine specificity of PCR assay using primers designed to amplify the ITS region in *Pseudoperonospora humuli*

Organism	Strain[a]	Host	Location	Amplification[b]
Pseudoperonospora humuli	BR82365	*Humulus lupulus*	Belgium	+
Pseudoperonospora humuli	SMK11608	*Humulus japonicus*	Korea, Kangnung	+
Pseudoperonospora humuli	SMK11675	*Humulus lupulus*	Korea, Suwon	+
Pseudoperonospora humuli	SMK19582	*Humulus japonicus*	Korea, Pyongchang	+
Pseudoperonospora humuli	HDM-039	*Humulus lupulus*	USA, Washington	+
Pseudoperonospora humuli	HDM-070	*Humulus lupulus*	USA, Idaho	+
Pseudoperonospora humuli	HDM-094	*Humulus lupulus*	USA, Washington	+
Pseudoperonospora humuli	HDM-101	*Humulus lupulus*	USA, Washington	+
Pseudoperonospora humuli	HDM-140	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-152	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-153	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-158	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-159	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-160	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-165	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-170	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-171	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-174	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-178	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-185	*Humulus lupulus*	USA, Oregon	+
Pseudoperonospora humuli	HDM-188	*Humulus lupulus*	USA, Idaho	+
Pseudoperonospora humuli	HDM-189	*Humulus lupulus*	USA, Idaho	+
Pseudoperonospora humuli	HDM-263	*Humulus lupulus*	Czech Republic	+
Pseudoperonospora humuli	HDM-264	*Humulus lupulus*	Czech Republic	+
Pseudoperonospora humuli	HDM-265	*Humulus lupulus*	Czech Republic	+
Pseudoperonospora humuli	HDM-266	*Humulus lupulus*	Czech Republic	+
Pseudoperonospora humuli	HDM-267	*Humulus lupulus*	Czech Republic	+
Pseudoperonospora cubensis	2279	*Impatiens irvingii*	Cameroon	+
Pseudoperonospora cubensis	SMK14235	*Citrullus vulgaris*	Korea, Chunchon	+
Pseudoperonospora cubensis	SMK11284	*Cucumis melo* var. *makuwa*	Korea, Kangnung	+
Pseudoperonospora cubensis	SMK15170	*Cucumis melo* var. *reticulatus*	Korea, Kimhae	+
Pseudoperonospora cubensis	SMK12174	*Cucumis sativus*	Korea, Kangnung	+
Pseudoperonospora cubensis	SMK18951	*Cucumis sativus*	Korea, Samchok	+
Pseudoperonospora cubensis	SMK13288	*Cucurbita moschata*	Korea, Kangnung	+
Pseudoperonospora cubensis	SMK19205	*Cucurbita moschata*	Korea, Chunchon	+
Pseudoperonospora cubensis	CDM-230	*Cucumis sativus*	USA, Michigan	+
Pseudoperonospora cubensis	CDM-231	*Cucumis sativus*	USA, Delaware	+
Pseudoperonospora cubensis	CDM-232	*Cucumis sativus*	USA, New Jersey	+
Pseudoperonospora cubensis	CDM-236	*Cucurbita pepo*	USA, New Jersey	+
Pseudoperonospora cubensis	CDM-244	*Cucumis sativus*	USA, North Carolina	+
Pseudoperonospora cubensis	CDM-245	*Cucumis sativus*	USA, Michigan	+
Pseudoperonospora cubensis	CDM-246	*Momordica charantia*	USA, North Carolina	+
Pseudoperonospora cubensis	CDM-247	*Cucumis pepo*	USA, New Jersey	+
Pseudoperonospora cubensis	CDM-248	*Cucumis pepo*	USA, North Carolina	+
Pseudoperonospora cubensis	CDM-251	*Cucumis sativus*	USA, Michigan	+
Pseudoperonospora cubensis	CDM-252	*Cucumis sativus*	USA, Ohio	+
Pseudoperonospora cubensis	CDM-253	*Cucumis sativus*	USA, North Carolina	+
Pseudoperonospora cubensis	CDM-254	*Cucumis sativus*	USA, North Carolina	+
Pseudoperonospora cubensis	CDM-255	*Cucumis sativus*	USA, Michigan	+
Pseudoperonospora celtidis	SMK17780	*Celtis sinensis*	Korea, Dongduchon	–
Pseudoperonospora celtidis	BPI 785661	*Celtis occidentalis*	USA, West Virginia	–
Pseudoperonospora cannabina	BPI 785653	*Cannabis sativa*	Latvia, Vidzeme	–
Pseudoperonospora cannabina	MZM71018	*Cannabis sativa*	Latvia, Riva	–
Pseudoperonospora urticae	WU 22947	*Urtica dioica*	Austria	–
Pseudoperonospora urticae	BPI 785732	*Urtica dioica*	Germany, Bavaria	–
Pseudoperonospora urticae	BPI 785733	*Urtica dioica*	Germany, Upper Bavaria	–
Pseudoperonospora urticae	BPI 785737	*Urtica dioica*	Sweden, Stockholm	–
Peronospora antirrhini	SDM1	*Misopates orontium*	USA, Oregon	–
Peronospora cristata	—	*Papaver somniferum*	Australia, Tasmania	–

Table 1 *Continued*

Organism	Strain[a]	Host	Location	Amplification[b]
Peronospora destructor	ODM1	*Allium cepa*	Australia, Tasmania	–
Peronospora farinosa f. sp. *chenopodii*	33	*Chenopodium quinoa*	Bolivia	–
Peronospora polygoni	WU 22917	*Polygonum aviculare*	Austria	–
Peronospora sparsa	—	*Rosa* sp.	USA, Oregon	–
Peronospora tabacina	BU06	*Nicotiana tabacum*	USA, North Carolina	–
Peronospora trifoli-repentis	WU22937	*Trifolium repens*	Austria	–
Peronospora viciae	—	*Pisum sativum*	Australia, Tasmania	–
Alternaria alternata	AA1	*Humulus lupulus*	USA, Washington	–
Alternaria solani	M1B4 SS AS	*Solanum tuberosum*	USA, Oregon	–
Alternaria solani	Mia13 SS AS	*Solanum tuberosum*	USA, Oregon	–
Botrytis cinerea	250	*Geranium* sp.	USA, Oregon	–
Burkholdaria gladioli	FP62	Unknown	USA, Oregon	–
Candida fumata	FP54	*Vitis vinifera*	USA, Oregon	–
Cryptococcus terreus	FP47	*Geranium* sp.	USA, Oregon	–
Erysiphe necator	—	*Vitis vinifera*	USA, Oregon	–
Fusarium avenaceum	F116	*Humulus lupulus*	USA, Oregon	–
Fusarium oxysporum f.sp. redolens	F47	*Zea mays*	USA, Oregon	–
Fusarium sambucinum	F119	*Humulus lupulus*	USA, Oregon	–
Fusarium sambucinum	F121	*Humulus lupulus*	USA, Oregon	–
Humulus lupulus	cv. Nugget	—	USA, Oregon	–
Phacidiopycnis pyri	ATCC MYA-3319	*Pyrus communis*	USA, Oregon	–
Phacidiopycnis washingtonensis	ATCC MYA-3321	*Malus sylvestris*	USA, Washington	–
Phomopsis tuberibora	PT-002	*Humulus lupulus*	USA, Oregon	–
Phragmidium violaceum	—	*Rubus* sp.	USA, Oregon	–
Phytophthora citricola	1858	*Pieris japonica*	USA, Oregon	–
Phytophthora citrophthora	1831	*Pieris japonica*	USA, Oregon	–
Phytophthora gonadopyides	1033	Soil	USA, Oregon	–
Phytophthora ramorum	07-1161-3-1	*Rhododendron* sp.	USA, Oregon	–
Podosphaera macularis	HPM-023	*Humulus lupulus*	USA, Washington	–
Podosphaera macularis	HPM-024	*Humulus lupulus*	USA, Washington	–
Podosphaera macularis	HPM-127	*Humulus lupulus*	Czech Republic	–
Podosphaera macularis	HPM-128	*Humulus lupulus*	Czech Republic	–
Pythium ultimum	ATCC 200006	*Nicotiana tabacum*	Canada, Ontario	–
Sclerotinia sclerotiorum	H05 WM	*Phaseolus vulgaris*	USA, Oregon	–
Verticillium albo-atrum	HVA-038	*Humulus lupulus*	USA, Oregon	–

[a] — Indicates no strain designation.

[b]Amplification of an amplicon of expected size with primers HDM04 and HDM07. + indicates amplification and – indicates no amplification. PCR conditions are as described in the text.

at least six hills each, placed arbitrarily along the border of the yard in each of three or four replications in a completely randomized design. Disease severity in the non-treated plots was assessed every 7 to 21 days throughout the season by counting the number of basal spikes per plant in plots not treated with fungicides.

A Rotorod spore trap was placed on the leeward edge of the hop yard near one of the non-treated plots. The spore trap was fitted with glass rods (2006 and 2007) or stainless steel rods (2008) lightly coated with silicon vacuum grease. Stainless steel rods were used in 2008 to prevent breakage that occasionally occurred with glass rods. All rods were 5 cm in length by 1 mm diameter, and were mounted 24 mm from the centre of the fixed sampling arm. With this configuration the spore traps sampled approximately 408 L of air per minute. The sampling rods were positioned at a height of 1·5 m above the ground and approximately 3 m from the nearest hop plant. Traps were not shielded from rain.

Rods were collected from the trap every 3 to 4 days. Depending on weather conditions and dustiness, at times the rods were mostly covered in dust particles and other environmental debris which could have reduced sampling efficiency (generally late spring to summer during extended periods of dry weather) (Bock & Cotty, 2006). However, during early spring sampling the rods were never overloaded with dust particles due to the generally wet, rainy conditions. Reduced sampling efficiency during certain times of year was also considered a minor source of sampling error since only presence or absence of *P. humuli* was determined by the PCR.

DNA was extracted from the organisms and materials on the rods using a MoBio UltraClean Soil DNA kit as described previously. One microlitre of the DNA extraction was used as template in the PCR assay. If the PCR was negative then it was repeated after spiking the mixture with 1 ng of genomic DNA of *P. humuli* to ensure amplification was not inhibited. PCR assays were also

conducted on a 1:10 dilution of the template and a 1:10 dilution of the template spiked with 1 ng of genomic DNA of *P. humuli* to overcome inhibitors and verify that amplification was possible after dilution. PCR products were periodically sequenced to verify that the DNA fragments were amplified from *P. humuli*. When the PCR assay was positive or negative, all days within that period when the rods were deployed were assumed to be positive or negative.

Sporangial density in the hop yard was measured with a Burkhard volumetric spore trap (Burkhard Manufacturing Company) operated continuously with the sampler orifice 1 m from the soil surface. Air flow was maintained at 10 L min^{-1} and was verified at least weekly. Sporangia matching the description of those of *P. humuli* were quantified by staining the Melinex tape with several drops of aniline blue and examining the entire tape under 160 × magnification.

Commercial hop yards

In 2007, two Rotorod spore traps were deployed in two commercial yards planted to the moderately resistant cv. Willamette (Haunold *et al.,* 1976) in Oregon (yards 1 and 2) and two yards planted to the susceptible cv. Tomahawk in Washington (yards 3 and 4). In 2008, the experiment was repeated in two hop yards in Oregon planted to cv. Nugget (yard 5) and cv. Super Galena (yard 6), which are susceptible and moderately resistant to downy mildew, respectively. Spore traps were placed along the leeward edge of the hop yards near poles to minimize the risk of interfering with equipment and grower field operations. The spore traps were fitted with sampling rods as described above. The sampling rods were positioned at a height of 1·5 m above the ground. Rods were collected from the trap every 5 to 7 days, and DNA was extracted as above.

Cooperating growers were asked to initiate their fungicide applications in response to the first detection of airborne inoculum in a portion of the yard (0·5 to 1·5 ha, depending on the yard) and initiate treatments to the remainder of the yard according to their standard management practices for downy mildew. Fungicides applied by the growers varied depending on their individual disease management programmes, and specific information on fungicide programmes is not presented to protect grower confidentiality. However, in each hop yard the fungicide treatments varied only by the timing of the first application made to the PCR-timed plots.

Disease severity (basal spikes per hill) was assessed every 7 to 14 days. Each yard was partitioned into strata of 20 rows in the standard and PCR-timed treatments. At least one row in each of the first two strata were selected arbitrarily and the number of downy mildew basal spikes was assessed on the first 75 to 100 plants in a single transect in each plot. To determine significant differences in disease severity between the plots, *t* tests (non-paired; $P < 0{\cdot}05$) were conducted for each sampling date and hop yard. Data were log-transformed prior to analysis to normalize variances.

Results

Pseudoperonospora humuli primer and PCR design

Alignment of the ITS region revealed regions of sequence heterogeneity suitable for design of primers for detection of *P. humuli*. However, due to the close relatedness of *P. cubensis* and *P. humuli* (Choi *et al.*, 2005), primers could not be designed in the ITS region that were specific to *P. humuli*. Primers HDM07 and HDM04 generated a product of 338 bp in all isolates of *P. humuli* and *P. cubensis* tested. However, this level of specificity was deemed suitable since cucurbit downy mildew does not occur at an appreciable level in hop production regions in the USA and successful amplification from hop tissue or air of a hop yard was assumed to indicate the presence of *P. humuli* with a low risk of a Type I error (i.e. false positive). A high level of specificity to *P. humuli* and *P. cubensis* was observed when the primers were tested against other related species and other organisms commonly associated with hop (Table 1).

The sensitivity of the PCR was 1 fg of genomic DNA of *P. humuli*. A single sporangium was detected on a silicon grease coated glass rod in 70% of PCR reactions, and 100% of reactions with five or more sporangia (Fig. 1). In the presence of 25 mg of soil, a PCR was successful in 20, 50, 90, 90 and 100% of reactions with 1, 5, 10, 100 and 500 sporangia, respectively. A 1:10 dilution of the extracted DNA with soil present resulted in the same proportion of successful amplifications.

Field validation

Experimental plots

In 2006, a single downy mildew lesion approximately 2 mm in diameter was detected on one plant on DOY 86 and the first sporangia of *P. humuli* were detected by the Burkhard spore trap on DOY 87. The pathogen was

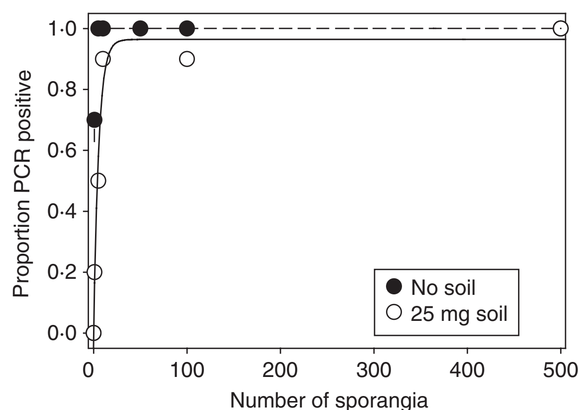

Figure 1 Sensitivity of a PCR assay for detection of *Pseudoperonospora humuli* in the presence and absence of soil. The solid and dashed lines are predicted sensitivity fit by nonlinear regression.

detected by PCR from the Rotorod trap on DOY 74, and then again on days 83 to 86 (Fig. 2a). The pathogen was detected intermittently by PCR up to the first application of carfentrazone-ethyl on DOY 101, and then was detected on all sampling dates until day 206. *Pseudoperonospora humuli* was detected intermittently from DOY 206 until sampling ceased on DOY 305. During DOY 206 to 305, sporangia were detected by the Burkhard spore trap on only four days (DOY 263, 265, 273 and 280).

Symptoms of downy mildew on a young shoot were observed on DOY 75 in 2007. On the following day sporulation was visible on the shoot, sporangia were detected using the Burkhard spore trap, and the PCR assay for *P. humuli* was positive. After this, *P. humuli* was detected by PCR for the entire season, except for days 194 –197 when amplification was inhibited (Fig. 2b)

In 2008, sporulation on a basal spike was observed on DOY 88 and inoculum was later subsequently detected by PCR on DOY 93 (Fig. 2c). The PCR was positive from DOY 93 to 200, corresponding to periods of inoculum detection in the Burkhard spore sampler and disease detection in the plots.

Commercial hop yards

2007 studies. In Oregon in 2007, a sporulating downy mildew spike was first detected in yard 1 on DOY 78. Disease severity remained low and downy mildew was not observed again until DOY 121 (Fig. 3a). *Pseudoperonospora humuli* was detected by PCR beginning on DOY 86. The pathogen was detected up to DOY 177 except for DOY 93–100 and 163–169.

Since the first detection of inoculum in this yard occurred prior to the chemical desiccation of spring growth that was applied on DOY 105, an application of 0·22 kg ha^{-1} metallic copper (Copper-Count-N, Mineral Research and Development) was made to the PCR-timed plots on DOY 120. This application corresponded to the first regrowth of shoots after spring pruning. In the grower timed plots, the first fungicide application (metallic copper) was made on DOY 154. Downy mildew severity was relatively low in the grower timed plots (less than mean of 0·13 basal spikes per hill), and downy mildew was not observed in the PCR-timed plots throughout the season. Downy mildew severity was significantly lower in the PCR-timed plots compared to the grower timing on four sampling dates.

In yard 2, a sporulating downy mildew spike was first observed on DOY 86, and then again on DOY 121 after plants had regrown following chemical desiccation (Fig. 3b). *Pseudoperonospora humuli* was detected by PCR beginning on DOY 86. Except for the sampling period of DOY 128 –135, *P. humuli* was detected up to DOY 176. Data for DOY 185 to 190 were excluded due to PCR inhibition.

Similarly to yard 1, the first detection of inoculum occurred prior to the chemical desiccation of spring growth. Therefore, an application of metallic copper was made to the PCR-timed plots on DOY 120. The first

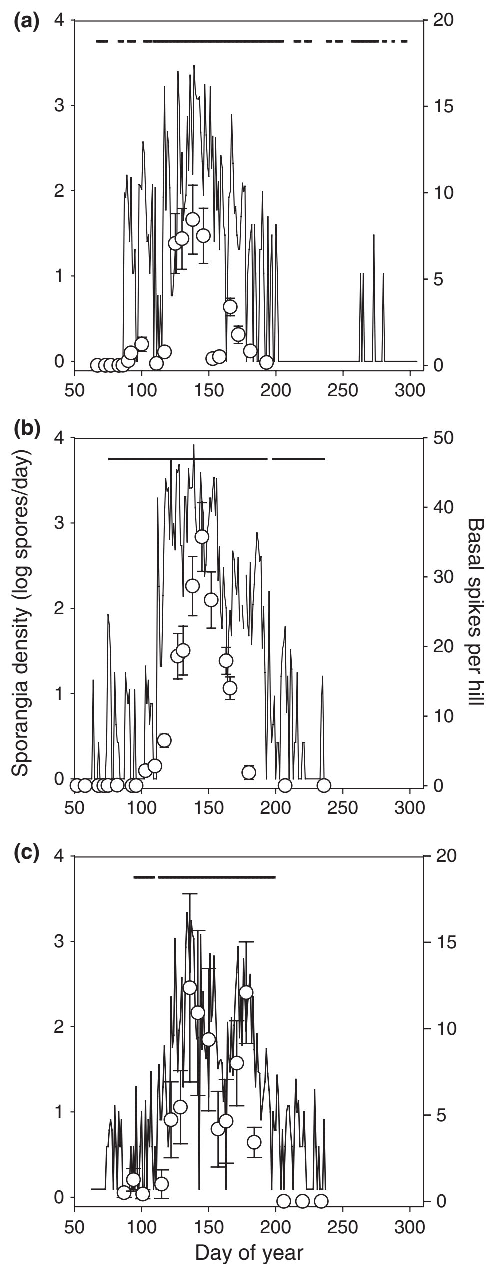

Figure 2 Severity of hop downy mildew recorded as systemically infected shoots (basal spikes), PCR detection, and density of sporangia of *Pseudoperonospora humuli* in air sampled from an experimental hop yard during 2006 (a), 2007 (b) and 2008 (c). Open circles = disease severity (± standard error of the mean). Solid line = sporangial density as measured by a Burkhard volumetric spore trap. Burkhard data is missing in Fig 2a for days 74 to 80 due to a malfunction of the spore trap. The line at the top of each figure indicates dates of positive PCR results for *P. humuli* from air samples.

application of metallic copper was made on DOY 154 in the grower timed plot. Downy mildew severity remained below a mean of 0·32 basal spikes per hill during the season. Downy mildew was significantly lower in the PCR-

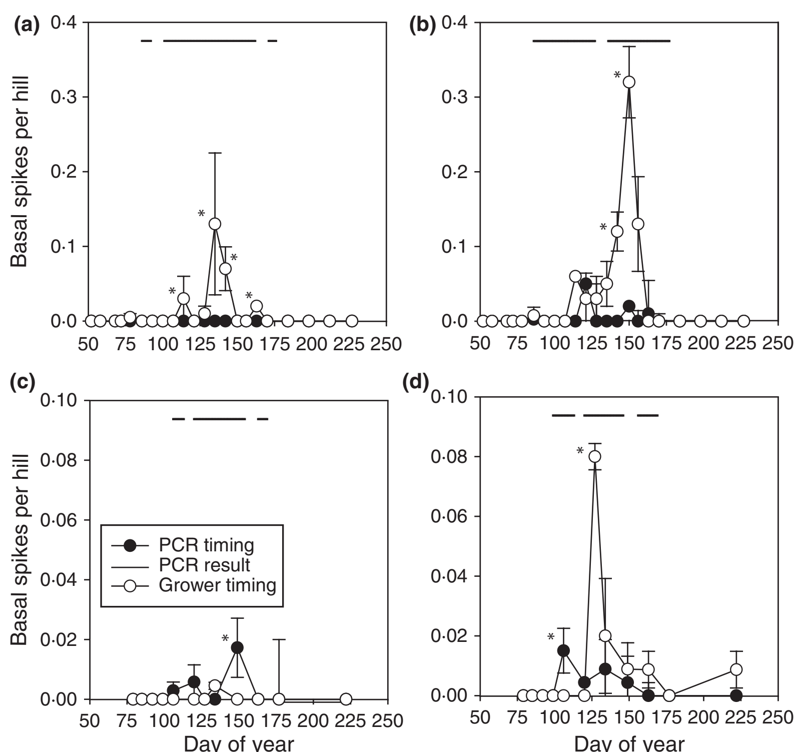

Figure 3 Severity of hop downy mildew recorded as systemically infected shoots (basal spikes) and PCR detection of *Pseudoperonospora humuli* in air sampled from four commercial hop yards in Oregon (a & b) and Washington (c & d) during 2007. PCR timing and grower timing refers to disease severity in plots where the growers' fungicide applications were initiated based on the first detection of *P. humuli* by PCR or the growers' standard practices, respectively. Asterisks denote significant differences based on a *t*-test ($P < 0·05$). The line at the top of each figure indicates dates of positive PCR results for *P. humuli* from air samples.

timed plots compared to the grower timing plots on two sampling dates.

In yard 3 in Washington, a sporulating downy mildew spike was detected on DOY 106, corresponding to the first PCR detection of *P. humuli* on that day (Fig. 3c). Inoculum of *P. humuli* was detected on most sampling dates when downy mildew was observed in the hop yard, with the exception of sampling periods DOY 156 –161 and 170 –175. Data from DOY 177 –189 were excluded due to PCR inhibition.

The first application to the PCR-timed plots occurred on DOY 132, and consisted of 1·2 kg ha^{-1} phosphorous acid (Genesis Foliar Phite, Genesis Agri Products). The first application according to the grower's standard timing occurred three days later (DOY 135). Downy mildew severity in either plot did not exceed 0·02 basal spikes per hill during the season, and differences in disease severity between the plots were observed only on DOY 149.

In yard 4 in Washington, a sporulating downy mildew spike was detected on DOY 106, which was seven days after the first detection of *P. humuli* by PCR on DOY 99 (Fig. 3d). Inoculum of *P. humuli* was detected on most sampling dates when downy mildew was observed in the hop yard, with the exception of sampling periods DOY 147 –154. PCR inhibition occurred on DOY 114 –199 and 177 –182.

The first application to the PCR-timed plots occurred on DOY 128 (phosphorous acid). The first application according to the grower's standard timing occurred three days later (DOY 131). Downy mildew severity was low in the plots throughout the season, and differences in disease severity between the plots were not observed consistently. Downy mildew severity was greater in the PCR-timed plots compared to the grower timing on DOY 106, but was significantly less in these plots on DOY 134.

2008 studies. In yard 5, sporulation was observed on DOY 127. Downy mildew severity was low and was detected on only two sampling dates throughout the season (Fig. 4a). *Pseudoperonospora humuli* was detected by PCR beginning on DOY 113, and then again on DOY 127 and intermittently up to DOY 190.

An application of metallic copper was made to the PCR-timed plots on DOY 122. The first application made in the grower-timed plots occurred on DOY 100. Downy mildew severity was similar in both plots, remaining below a mean of 0·1 basal spikes per hill during the season.

In yard 6, a downy mildew spike was first observed on DOY 127. Downy mildew severity was low and was not observed again (Fig. 4b). *Pseudoperonospora humuli* was detected by PCR beginning on DOY 113, 14 days prior to detection of disease in this yard.

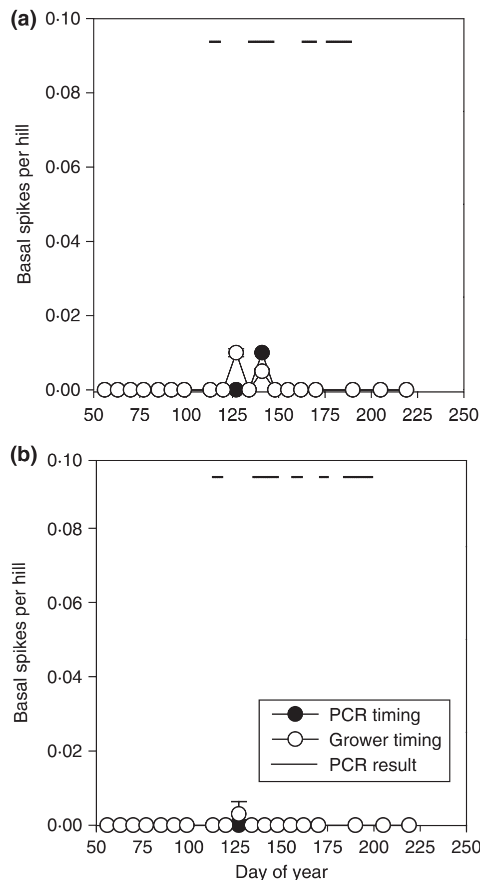

Figure 4 Severity of hop downy mildew recorded as systemically infected shoots (basal spikes) and PCR detection of *Pseudoperonospora humuli* in air sampled from two commercial hop yards in Oregon during 2008. PCR timing and grower timing refers to disease severity in plots where the growers' fungicide applications were initiated based on the first detection of *P. humuli* by PCR or the growers' standard practices, respectively. The line at the top of each figure indicates dates of positive PCR results for *P. humuli* from air samples.

An application of metallic copper (Copper-Count-N) was made to the PCR-timed plots on DOY 120, and in the grower-timed plots on DOY 100. Downy mildew severity was similar in both plots, remaining below a mean of 0·1 basal spikes per hill during the season.

Discussion

The timing of initial fungicide applications for control of downy mildew has become more critical in the USA due to the emergence of insensitivity to phenylamide (i.e. metalaxyl and mefenoxam) and phosphonate fungicides (i.e. fosetyl-Al) in strains of *P. humuli* (Klein, 1994; Nelson *et al.*, 2004; Gent *et al.*, 2008). Relatively widespread insensitivity to both of these chemistries in Oregon, northern Idaho, and certain hop yards in Washington necessitate more intensive applications of other fungicides (e.g. cymoxanil, copper) with shorter residual activities (Hunger & Horner, 1982; Hellwig *et al.*, 1991; Nelson *et al.*, 2004; Gent *et al.*, 2008). Current management recommendations are based on the assumption that inoculum is always present, since quantitative monitoring of sporangial density is not feasible with hop farms in the USA. An inoculum detection approach to timing fungicide applications, as demonstrated in these studies, could help to reduce unnecessary early season applications under low disease pressure or enhance control under more moderate disease pressure.

During the nine location-years of validation, PCR detection of inoculum of the pathogen in air samples occurred no later than 8 days after the appearance of trace levels of disease and/or airborne spores. Inoculum was detected on average 4·5 days before (range −8 to 14 days) the first appearance of basal spikes in six commercial hop yards, or 1·3 days after (range −5 to 1 days) sporangia were detected in a Burkhard volumetric spore sampler in experiment plots.

There were several sampling periods when the PCR assay was positive but sporangia of *P. humuli* were not detected in the Burkhard spore trap. Presumably these discrepancies could be attributed to non-specific amplification during the PCR, misidentification of pathogen in the Burkhard spore trap, or the lower sampling volume of the Burkhard spore trap (10 L min^{-1}) compared to the Rotorod spore trap (>400 L min^{-1}). The PCR assay provides a measure of the presence of DNA of *P. humuli* (and *P. cubensis*) but provides no information on the source of inoculum or its viability. Detection of oosporic inoculum, particularly during extended dry, dusty conditions, could be another explanation for positive PCR results when sporangia of *P. humuli* were not detected by the Burkhard spore trap. Oospores of *P. humuli* are formed readily in diseased leaves and cones (Royle & Kremheller, 1981), and potentially wind-blown soil in hop yards could contain oospores that could be trapped on the rods and subsequently detected by PCR.

In commercial hop yards, use of the PCR to initiate the first fungicide application for downy mildew enhanced disease control in two hop yards in Oregon in 2007 when PCR-aided application were made 34 days before the growers' timing. Conversely, use of the PCR allowed the growers in Oregon in 2008 to delay their first fungicide application 20 to 22 days, resulting in the saving of one unnecessary fungicide in both instances. Although disease control and the date of the first fungicide application were similar between the PCR and grower timing in Washington in 2007, the PCR assay essentially validated the growers' fungicide timing. That is, the growers' timing was within three days of the first detection of inoculum by the PCR. Taken together, these results indicate that the efficiency and efficacy of downy mildew management can be improved when control measures are timed according to first detection of inoculum.

Although quantitative information on inoculum density would be most informative, knowledge of the presence or absence of inoculum is appropriate for: (i) initiating control measures early in the season; and (ii)

terminating control measures later in the season. Information on simple presence of inoculum is likely to overestimate disease risk (type I error) and would tend to result in more liberal recommendations for the need for disease control measures (less type II errors). Given that agricultural producers are risk averse (Pannell, 1991; Madden *et al*., 2007), knowledge of inoculum presence/absence would tend to decrease the more serious type II error of not applying a control measure when it is warranted.

The specificity of the assay to both *P. humuli* and *P. cubensis* could be problematic in certain situations where *P. humuli* is not present in a hop yard but inoculum of *P. cubensis* is present. Such situations seem improbable since cucurbits are not grown on an extensive scale in hop producing regions in the USA, downy mildew of cucurbits occurs rarely in the western USA, and epidemics of hop downy mildew begin months before cucurbit crops are planted or many weedy cucurbit species emerge.

However, *P. humuli* and *P. cubensis* may be synonymous as suggested by Choi *et al*. (2005) or perhaps *P. humuli* is a host specific strain of *P. cubensis*. Detailed studies on the host range of these pathogens and sequencing of genes with higher phylogenetic resolution than ITS are needed to address these issues. Sequencing of the *cox* gene cluster, *nadh* and beta tubulin genes have been conducted from multiple isolates of *P. humuli* and *P. cubensis* (data not presented) and have detected several single nucleotide polymorphisms that are conserved between the pathogens. Additional research is needed to develop a PCR assay more specific to *P. humuli*. However, the current PCR assay may be of use for detection of *P. cubensis* in air samples in regions where hops are not produced, such as the southern USA, as a component of a warning system for cucurbit downy mildew risk (Main *et al*., 2001).

It is likely that a PCR assay could be useful for confirmation of *P. humuli* in symptomless tissues such as hop cones or shoots when sporangia of the pathogen are not present. Diseased shoots can be identified readily by the presence of profuse dark purple to black sporangia produced on abaxial leaf surfaces, although low humidity and temperature can suppress sporangial production (Johnson & Skotland, 1985) and complicate disease diagnosis. In preliminary studies it has been confirmed that detection of *P. humuli* by the PCR assay is possible in latently infected leaves, cones and shoots. Potentially, the PCR could also be used to verify planting materials free of *P. humuli*, which is an important source of initial inoculum in newly planted hop yards (Skotland & Johnson, 1983).

The discussion of economic benefits presented by Falacy *et al*. (2007) for a similar PCR-based approach to air sampling are also relevant for hop downy mildew. While most farms clearly would not have the facilities or infrastructure available for sample processing, it is envisioned that simple impaction spore traps could be deployed and maintained by private crop consultants or individual growers, and a DNA extraction protocol and PCR assay could be transferred to existing analytical laboratories and commercialized on a fee-for-service basis. It is also anticipated that the air sampling approach used in these studies could be conducted routinely in individual hop yards or in representative 'sentinel' plots to issue early season disease risk warnings on a regional basis.

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