Detection and Quantification of *Bremia lactucae* by Spore Trapping and Quantitative PCR

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Accepted for publication 24 May 2016.

ABSTRACT

Kunjeti, S. G., Anchieta, A., Martin, F. N., Choi, Y. J., Thines, M., Michelmore, R. W., Koike, S. T., Tsuchida, C., Mahaffee, W., Subbarao, K. V., and Klosterman, S. J. 2016. Detection and quantification of *Bremia lactucae* by spore trapping and quantitative PCR. *Phytopathology* 106:1426-1437.

Bremia lactucae is an obligate, oomycete pathogen of lettuce that causes leaf chlorosis and necrosis and adversely affects marketability. The disease has been managed with a combination of host resistance and fungicide applications with success over the years. Fungicide applications are routinely made under the assumption that inoculum is always present during favorable environmental conditions. This approach often leads to fungicide resistance in *B. lactucae* populations. Detection and quantification of airborne *B. lactucae* near lettuce crops provides an estimation of the inoculum load, enabling more judicious timing of fungicide applications. We developed a quantitative polymerase chain reaction (qPCR)-based assay

using a target sequence in mitochondrial DNA for specific detection of *B. lactucae*. Validation using amplicon sequencing of DNA from 83 geographically diverse isolates, representing 14 *Bremia* spp., confirmed that the primers developed for the TaqMan assays are species specific and only amplify templates from *B. lactucae*. DNA from a single sporangium could be detected at a quantification cycle (C_q) value of 32, and C_q values >35 were considered to be nonspecific. The coefficient of determination (R^2) for regression between sporangial density derived from flow cytometry and C_q values derived from the qPCR was 0.86. The assay was deployed using spore traps in the Salinas Valley, where nearly half of U.S. lettuce is produced. The deployment of this sensitive *B. lactucae*-specific assay resulted in the detection of the pathogen during the 2-week lettuce-free period as well as during the cropping season. These results demonstrate that this assay will be useful for quantifying inoculum load in and around the lettuce fields for the purpose of timing fungicide applications based on inoculum load.

Bremia lactucae (Regel) is an obligate biotrophic oomycete that causes downy mildew of lettuce, a disease of economic importance worldwide. Infection on outer leaves results in yield loss and severe outbreaks of the disease renders the crop unmarketable (Van Bruggen and Scherm 1997). Foliar tissue infected by downy mildew also provides an entry site for opportunistic plant pathogens and human enteric pathogens such as *Escherichia coli* and *Salmonella enterica* (Seaman 2015; Simko et al. 2015) that further accentuate postharvest crop losses.

Sporangia from other fields of cultivated lettuce or wild lettuce plants (*Lactuca serriola* L.) and, more rarely, oospores in plant debris can serve as primary inoculum sources (Van Hese et al. 2016) and can infect lettuce from seedling stage to mature plants (Mieslerova et al. 2013). Symptoms of lettuce downy mildew typically occur as angular, pale-yellow patches delineated by leaf veins. Signs of the pathogen are readily observed as downy white

growth, typically on the abaxial side of the leaves (Mieslerova et al. 2013). Sporangiophores emerge through the stomata and produce large numbers of asexual sporangia that land on uninfected leaf tissue to cause new infections. Sporangia are also dispersed by water splash and air currents to healthy plants (Seaman 2015). The secondary disease spread occurs by airborne sporangia (Fletcher 1976).

Lettuce production is highly concentrated in several production areas in California and Arizona that rotate with the seasons. The central coast of California produces 32.2% of lettuce in the United States, a crop valued at over \$2.4 billion in 2014 (FAOSTAT 2014; Monterey County Agricultural Commissioner's Office 2014). The crop is grown throughout the year, except for a brief lettuce-free period in December each year. Downy mildew epidemics can occur and cause severe damage whenever and wherever lettuce is grown. The prevalent weather conditions in the coastal valleys with temperatures $<23^{\circ}\text{C}$ and relative humidity $>85\%$ are favorable for the development of downy mildew of lettuce (Su et al. 2004).

Species of *Bremia* are highly host specific and mostly limited to the same host plant species or genus (Choi and Thines 2015; Choi et al. 2011; Mieslerova et al. 2013; Thines et al. 2010; Voglmayr et al. 2004), similar to the situation described for other downy mildews (Choi et al. 2015). Some researchers have sought to determine the differences between species of *Bremia* (Riethmüller et al. 2002; Shin and Choi 2006; Voglmayr et al. 2004). Choi and Thines (2015) used multigene phylogenies to demonstrate the distinctiveness of *Bremia* spp. from 57 hosts into clades representing

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures and two supplementary tables are published online.

<http://dx.doi.org/10.1094/PHYTO-03-16-0143-R>

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species with a specificity, mostly on the host genus level or below, with *B. lactucae* from two hosts (*L. sativa* and *L. serriola*) grouped into the same clade (Choi and Thines 2015).

Downy mildew of lettuce is currently managed by a combination of genetic resistance, cultural practices, and chemical protection. Monogenic resistance from wild and cultivated lettuce has provided ephemeral resistance (Crute 1992; Parra et al. 2016); however, new *B. lactucae* races have emerged that have rendered these genes ineffective, thereby complicating resistance breeding efforts that, in turn, require additional disease management practices (Crute and Norwood 1981; Gianessi and Reigner 2005; Grimault 2015). Fungicide applications against downy mildew may provide significant improvements in marketable yield (Raid and Datnoff 1989). However, the deregistration of compounds due to environmental concerns and the emergence of fungicide-resistant isolates of *B. lactucae* have led to reduced options for chemical management (Brown et al. 2004; Crute et al. 1987; Isaac 1999; Schettini et al. 1991; Wicks et al. 1994), although some of these compounds have been replaced by fungicides with new chemistries. Chemical management of downy mildew is most effective when contact fungicides are applied before infection (Zijlstra et al. 2011). However, currently available conventional polymerase chain reaction (PCR) methods and data are not adequate for developing and implementing a forecast or warning model to guide fungicide applications for downy mildew of lettuce.

To aid in the detection of particular plant pathogens, several immunological and nucleic acid-based techniques have been developed (Ward et al. 2004). Conventional PCR, nested PCR, and real-time PCR are nucleic acid-based techniques commonly deployed to detect DNA of pathogens. PCR-based detection of *Pseudoperonospora humuli* in air samples from commercial hop yards has been useful to time fungicide applications (Gent et al. 2009) and nested PCR improved the sensitivity and specificity of this assay (Foster et al. 2002; Mutasa et al. 1993; Schesser et al. 1991). However, neither method is quantitative. Detection and quantification of plant-pathogenic fungi and oomycetes were simplified considerably with the emergence of real-time quantitative PCR (qPCR). The application of qPCR can be conducted using either nonspecific DNA-binding dyes (SYBR Green) or fluorescent probes that are specific to the target DNA (Wittwer et al. 1997). The DNA targets that have been useful for detection and quantification of plant-pathogenic oomycetes include the internal transcribed spacers (ITS) or intergenic spacer of the ribosomal DNA (rDNA), alternative nuclear genomic regions (i.e., β -tubulin), mitochondrial DNA, and random DNA fragments (i.e., random amplified polymorphic DNA or amplified fragment length polymorphism) (Bilodeau et al. 2007, 2014; Demontis et al. 2008; Klosterman et al. 2014; Martin et al. 2012; Schena et al. 2013; Schroeder et al. 2013). Previous work on *B. lactucae* has only involved detection of the pathogen using the ITS region (Mota et al. 2011).

Development of a specific qPCR procedure for detection and quantification of *B. lactucae* sporangia from spore trap samples represents the initial step in designing a disease forecasting system for downy mildew of lettuce. The ability to detect and quantify airborne sporangia before the occurrence of disease outbreaks, or at their initial onset, may be helpful for more effective timing of fungicide applications for optimal disease management, and reduce the number of fungicide applications during lettuce production and, thereby, reduce or delay the development of fungicide insensitivity (Klosterman et al. 2014; Mahaffee and Stoll 2016). Spore sampling systems, in combination with DNA amplification using conventional or real-time PCR-based methods, have been deployed successfully for the detection of several airborne plant pathogens (Calderon et al. 2002; Carisse et al. 2008, 2009; Falacy et al. 2007; Fraaije et al. 2005; Klosterman et al. 2014; Rogers et al. 2009; Schena et al. 2013; Schweigkofler et al. 2004; Thiessen et al. 2016; Urbez-Torres et al. 2010; West and Kimber 2015). These studies

highlight the potential utility of spore trap and DNA-based assays in the analyses of airborne inoculum dispersal for economically important plant pathogens. Yet no method for rapid detection and quantification of *B. lactucae* is available, nor have spore traps been deployed in central coastal California or elsewhere for detection of this pathogen.

The objectives of this study were to (i) develop a sensitive, *B. lactucae*-specific qPCR assay for detection and quantification of the pathogen; (ii) quantify *B. lactucae* DNA extracted from impaction spore trap samples collected in the Salinas Valley of California during lettuce production and during a lettuce-free period; and (iii) develop an internal control (IC) for the assay to determine whether PCR inhibitors in DNA extracted from spore traps influence the amplification efficiency of the *B. lactucae* target sequence and, thus, the accuracy of results.

MATERIALS AND METHODS

Sequence analyses for assay development. Mitochondrial genomes have been assembled for 10 species representing six genera of downy mildews, including a total of 69 isolates of *B. lactucae* (F. Martin and R. Micheltore, unpublished). Comparative genomics identified a unique 861-bp putative open reading frame (*orf286*) of unknown function in *B. lactucae* that was not present in other oomycete taxa (F. Martin, unpublished). A 95-bp region close to the 5' end of the putative open reading frame was selected for design of the *B. lactucae*-specific marker but no significant similarity of this sequence was detected in GenBank using BLAST searches.

Collection of sporangia of *B. lactucae*. Sporangia of *B. lactucae* (isolate C14C1452) were inoculated onto 7-day-old lettuce seedlings placed on a sterile filter paper in Magenta boxes. The Magenta boxes with lettuce seedlings were incubated at 15°C for 6 days to sporulation. At 6 days postinoculation, the infected cotyledons were transferred into a clean magenta box using flame-sterilized tools in a biological hood. Distilled water (15 ml) was poured over the cut cotyledons and swirled to remove the spores. The spore suspension was then filtered through cheesecloth into a 15-ml tube and centrifuged at $3,400 \times g$ for 20 min. The supernatant was decanted, leaving a 0.5-ml volume of distilled water and pellet in the tube.

Assay specificity using conventional PCR. Specificity testing of the primer set 1F (5'-GGAGTCATTGTTTGATTTAACT-3') and 95R (5'-AACTCAGAGCTAGATTACCA-3') was used to amplify sequencing template from DNA extracted from 71 isolates of *Bremia* spp. representing a worldwide distribution of geographic locations (Table 1). The DNA for this purpose were extracted using the BioSprint 96 DNA Plant Kit (Qiagen) on a KingFisher Flex (Thermo Scientific) robot. The amplification master mix for each reaction contained 0.4 μ M each species-specific primer pair, 0.5 U of Mango *Taq* polymerase (Bioline GmbH), 2.0 mM $MgCl_2$, 0.2 mM dNTP, 1 $\times$ buffer, and 0.8 μ g of bovine serum albumen, with PCR cycling conditions set at 95°C for 10 min and 40 cycles at 95°C for 15 s and 63°C for 60 s, in a reaction volume of 20 μ l on the Eppendorf Mastercycler (Eppendorf GmbH). The size of the amplified products was confirmed by electrophoresis on a 1.2% agarose gel.

Primer and probe design for TaqMan assays. Species-specific primers and probes were designed to amplify a 86-bp portion of *orf286*. Similar to the approach that Bilodeau et al. (2014) used to enhance amplification efficiency, a 5' flap was added to the primers (lowercase letters) F17 (5'-aataaatcataaGTCATTGTTTGATTTAACT-3') and R18 (5'-aataaatcataaGAGCTAGATTACCA-3'). The following parameters were used for designing the TaqMan probe from the central portion of the amplicon: melting temperature 10°C higher than the primers, 15 to 30 bp in length, and the total number of Gs or Cs in the last five nucleotides at the 3' end of the primer not exceeding two (Bilodeau et al. 2012); BL_probe = 5'-ATCAATAGAATGTCCCACTGCAAT-3'. The 5' end

of the probe was labeled with fluorescein and the 3' end with Black Hole Quencher-1 (Biosearch Technologies, Inc.) (Supplementary Fig. S1A).

Flow cytometry-based counting and sorting of *B. lactucae* sporangia. The BD Influx cell sorter (BD Biosciences) was used to generate 14 different counts of sporangia (1, 5, 10, 15, 20, 25, 30, 35, 40, 50, 100, 300, 600, and 1,000 sporangia), with 24 replicates each for 1 and 5 sporangia and 12 replicates each for 10 through 1,000 sporangia. The fresh spore pellet was resuspended in 5 ml of 10× phosphate-buffered saline solution (pH 4.1 to 4.7) (Corning Cellgro). This solution was filtered through a 5-ml sterile polystyrene round-bottom tube with a cell strainer cap (30-micron mesh) (REF 352235; Falcon). Before loading onto the BD Influx cell sorter, the filtrate was transferred to a sterile polypropylene round-bottom tube (REF 352063; Falcon). Sporangia of each count were dispensed directly into 24-well plates (CytoOne) containing 300 µl of extraction buffer (PL1, Nucleospin Plant II kit; Machery-Nagel) in each well. The BD influx parameters included forward and side scatter at 488 nm and additional fluorescence detectors with an excitation wavelength of 532 nm and collection window of 579/34

(approximately 562 to 596 nm) while the second used an excitation wavelength of 488 nm and collection window of 513/17 (approximately 505 to 521 nm). Immediately after sorting, the number of 1, 5, 10, and 25 sporangia was confirmed by counting under a compound scope; this was repeated at least eight times for each sporangial count.

DNA extraction, assay efficiencies, and limits of detection. The *B. lactucae* sporangia DNA extraction procedure was a modification of that reported by Bernatzky and Tanksley (1986). The sporangia were homogenized in a 2-ml microcentrifuge tube containing approximately 200 µl of Rainex-treated beads with 0.5 ml of 2× extraction buffer (100 mM Tris-HCL [pH 8.0], 1.4 M NaCl, 20 mM EDTA, 2% [wt/vol] cetyltrimethylammonium bromide, and β-mercaptoethanol at 20 µl/ml) by vortexing for 2 min and transferring the liquid to a fresh 2-ml tube. The used beads were rinsed with 0.5 ml of 2× extraction buffer and then transferred to the 2-ml tube. Subsequently, RNase (20 µl/ml) was added and incubated at 65°C for 30 min. An equal volume of 1:1 phenol/chloroform was added, mixed, and then centrifuged at maximum speed (5,200 × g) for 15 min. The aqueous phase was retained and mixed with an equal volume of 24:1 chloroform/isoamyl

TABLE 1. Specificity test for the detection of *Bremia lactucae* using mtDNA from different *Bremia* spp. of multiple plant hosts and various geographic locations^a

Species	Host plant	Herb. no.	Year ^b	Geographic origin	Isolate ID	PCR ^c	qPCR ^d
<i>B. centaureae</i> s.l.	<i>Centaurea cyanus</i>	GLM63037	2004	Germany, Sachsen	2049	–	NT
<i>B. centaureae</i> s.l.	<i>C. cyanus</i>	GLM78288	1999	Germany, Sachsen-Anhalt	2051	–	NT
<i>B. centaureae</i> s.l.	<i>C. cyanus</i>	GLM64168	2004	Germany, Sachsen-Anhalt	2053	NT	ND500
<i>B. centaureae</i> s.l.	<i>C. cyanus</i>	GLM75867	2005	Germany, Sachsen-Anhalt	2052	NT	ND500
<i>B. cirsi</i> s.l.	<i>Cirsium arvense</i>	GLM73818	2001	Germany, Sachsen-Anhalt	2082	–	NT
<i>B. cirsi</i> s.l.	<i>C. arvense</i>	GLM65673	2004	Germany, Sachsen-Anhalt	2072	NT	ND5
<i>B. cirsi</i> s.l.	<i>C. arvense</i>	GLM65724	2004	Germany, Sachsen-Anhalt	2077	NT	ND500
<i>B. cirsi</i> s.l.	<i>C. arvense</i>	GLM75827	2005	Germany, Sachsen-Anhalt	2081	–	NT
<i>B. elliptica</i>	<i>Lactuca indica</i> var. <i>laciniata</i>	KUS-F18913	2002	South Korea, Chunchon	2494	–	ND5
<i>B. elliptica</i>	<i>L. indica</i> var. <i>laciniata</i>	KUS-F20287	2004	South Korea, Chungju	2492	–	NT
<i>B. elliptica</i>	<i>L. indica</i> var. <i>laciniata</i>	KUS-F20331	2004	South Korea, Heongseong	2493	–	ND500
<i>B. lactucae</i>	...	...	1970s	Australia	AM	+	NT
<i>B. lactucae</i>	...	...	1970s	Czech Republic	CS12	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	1978	England, Cambridge	IM25P11 ^e	+	NT
<i>B. lactucae</i>	...	...	1970s	Finland	SF3	+	NT
<i>B. lactucae</i>	...	...	1970s	Finland	SF5	+	NT
<i>B. lactucae</i>	<i>L. serriola</i>	GLM86436	2006	Germany, Sachsen	2124	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	GLM77941	1998	Germany, Sachsen-Anhalt	2117	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	GLM75924	2005	Germany, Sachsen-Anhalt	2118	+	NT
<i>B. lactucae</i>	<i>L. sativa</i> var. <i>capitata</i>	GLM66916	1982	Germany, Sachsen-Anhalt	2119	+	NT
<i>B. lactucae</i>	<i>L. serriola</i>	GLM74487	2003	Germany, Sachsen-Anhalt	2125	+	NT
<i>B. lactucae</i>	<i>L. serriola</i>	GLM76900	2000	Germany, Sachsen-Anhalt	2126	+	NT
<i>B. lactucae</i>	<i>L. serriola</i>	GLM76306	2002	Germany, Sachsen-Anhalt	2127	+	NT
<i>B. lactucae</i>	<i>L. serriola</i>	GLM76317	2002	Germany, Sachsen-Anhalt	2128	+	NT
<i>B. lactucae</i>	<i>L. serriola</i>	GLM75746	2005	Germany, Sachsen-Anhalt	2129	+	NT
<i>B. lactucae</i>	<i>L. serriola</i>	GLM79348	2007	Germany, Sachsen-Anhalt	2130	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	1970s	Israel	IL4	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	1970s	Netherlands	NL6	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	1970s	Sweden	S1	+	NT
<i>B. lactucae</i>	...	...	1970s	Switzerland	CG1	+	NT
<i>B. lactucae</i>	...	...	1970s	United Kingdom	R60	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	Arizona	A14C1430	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	Arizona	A14C1432	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	Arizona	A14C1438	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	Arizona	A14C1460	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	Arizona	A14C1474	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	Arizona	A14C1476	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2013	California	C13C1427	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1459	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1464	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1469	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1500	+	NT

(continued on next page)

^a Abbreviations: s.l. = sensu lato, NT = not tested, ND500 = not detected (500 pg of DNA was used), and ND5 = not detected (5 pg of DNA was used).

^b Year of collection.

^c Conventional polymerase chain reaction (PCR); + = PCR product amplified and – = no PCR product.

^d Quantitative PCR.

^e IM25P11 is a single-spore derivation of IM25.

^f For the qPCR, 2 pg of DNA was used.

^g DNA could not be quantified using Qubit HS assay kit.

alcohol, mixed, and then centrifuged at $5,200 \times g$ for 15 min. The aqueous phase was mixed with 0.7 volumes of isopropanol and incubated at -23°C for 1 h. The precipitate was centrifuged at $20,800 \times g$ for 30 min at 4°C . The resulting DNA pellet was washed in 70% ethanol, dried, and then dissolved in 100 μl of 10-mM Tris-HCL. TaqMan assay linearity and efficiency was determined using six serial log 10-fold dilutions from 2 ng to 20 fg of DNA (isolate C14C1492; BI-DNA assay). Similarly, DNA for a standard curve was extracted independently from each of the above 24-well plates (different sporangial counts; isolate C14C1452), as described by Klosterman et al. (2014), with an initial vortex step of 10 min.

Evaluation of qPCR inhibition. Impaction spore trap sample rods obtained from the field were coated with silicon vacuum grease and often had soil particles and insect parts, in addition to pathogen spores. To evaluate whether these components influence amplification efficiency, amplification of purified *B. lactucae* target sequences was evaluated in the presence or absence of sample rod extract. DNA was extracted from sample rods that were deployed in pathogen-free areas (Klosterman et al. 2014) and eluted in 65 μl of elution buffer. Extracted DNA from different *B. lactucae* sporangial counts and 200 fg of purified DNA were combined with 1 μl of the eluate from sample rods collected from pathogen-free fields for qPCR analyses (BI-Gr assay). To calculate the amount of PCR inhibition from spore trap samples, the quantification cycle (C_q) values obtained from *B. lactucae* DNA amplification without sample rod extract present were subtracted from those obtained in the presence of sample rod extracts.

Spore sample collection and quantification. Spore sample collections and DNA extractions from impaction spore samplers were conducted as described previously (Klosterman et al. 2014), unless specified otherwise. For this study, spore traps were placed at three separate sites in California (King City, Castroville, and the United States Department of Agriculture–Agricultural Research Service [USDA-ARS], Salinas). Two spore traps were set in each of the four locations near spinach and lettuce fields from April through July 2015 near King City. These samples were collected on Monday and Friday of every week. Another set of two samplers was placed in Castroville, next to a commercial lettuce field, and sampled from August through September 2015. Also, four samplers were set up each winter season in 2013–14 and 2014–15 at a USDA-ARS plot maintained at the Salinas location, designated as USDA 2013-14 and USDA 2014-15, and spanned the lettuce-free period (7 to 21 December). The spore samples from the Castroville and USDA 2013-14 and USDA 2014-15 locations were collected three times a week (Monday, Wednesday, and Friday).

Real-time PCR with purified DNA and spore trap samples. All qPCR experiments in this study were performed in 384-well plates using a LightCycler 480 II (Roche Diagnostics). The qPCR were run in triplicate using one DNA extraction per spore trap. The qPCR volume of 12 μl contained 200 nM probe, 200 nM each primer, $1\times$ real master mix (5 PRIME), and 1 μl of DNA. Depending on quantities available, we used 500, 5, or 2 pg of DNA for specificity tests. The LightCycler 480 II with LightCycler 480 software (release 1.5.0) and the Absolute Quant/Fit Point analysis was used to conduct the assays with a reaction profile of 10 min at 95°C for

TABLE 1. (continued from preceding page)

Species	Host plant	Herb. no.	Year ^b	Geographic origin	Isolate ID	PCR ^c	qPCR ^d
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1504	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1508	+	NT
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1492	+	28.7075 ^f
<i>B. lactucae</i>	<i>L. sativa</i>	...	2014	California	C14C1452	NT	29.9500 ^f
<i>B. lapsanae</i>	<i>Lapsana communis</i>	GLM62892	2004	Germany, Sachsen-Anhalt	2140	NT	ND500
<i>B. lapsanae</i>	<i>L. communis</i>	GLM62909	2004	Germany, Sachsen-Anhalt	2143	–	NT
<i>B. lapsanae</i>	<i>L. communis</i>	GLM65736	2004	Germany, Sachsen-Anhalt	2147	–	NT
<i>B. lapsanae</i>	<i>L. communis</i>	GLM75913	2005	Germany, Sachsen-Anhalt	2144	NT	35.6602 ^g
<i>B. microspora</i>	<i>Ixeris chinensis</i>	KUS-F20256	2004	South Korea, Chunchon	2497	–	NT
<i>B. microspora</i>	<i>I. chinensis</i>	KUS-F20266	2004	South Korea, Chunchon	2498	–	NT
<i>B. microspora</i>	<i>I. polycephala</i>	KUS-F18823	2002	South Korea, Chunchon	2501	–	NT
<i>B. microspora</i>	<i>I. chinensis</i>	KUS-F18854	2002	South Korea, Namyangju	2502	–	ND5
<i>B. microspora</i>	<i>I. chinensis</i>	KUS-F20212	2004	South Korea, Namyangju	2500	–	ND500
<i>B. microspora</i>	<i>I. chinensis</i>	KUS-F17175	1999	South Korea, Namyangju	2495	–	NT
<i>B. microspora</i>	<i>I. chinensis</i>	KUS-F17225	1999	South Korea, Suwon	2499	–	NT
<i>B. microspora</i>	<i>I. polycephala</i>	KUS-F19288	2002	South Korea, Wonju	2496	–	NT
<i>B. ovata</i>	<i>Crepidiastrum denticulata</i>	KUS-F21509	2005	South Korea, Gapyeong	2504	–	NT
<i>B. ovata</i>	<i>Youngia japonica</i>	KUS-F19357	2002	South Korea, Jeju	2507	–	ND500
<i>B. ovata</i>	<i>Y. japonica</i>	KUS-F17172	1999	South Korea, Namyangju	2506	–	NT
<i>B. ovata</i>	<i>Y. japonica</i>	KUS-F20748	2004	South Korea, Namyangju	2505	–	ND500
<i>B. ovata</i>	<i>C. denticulata</i>	KUS-F19258	2002	South Korea, Yangpyeong	2503	–	NT
<i>B. picridis</i>	<i>Picris hieracioides</i>	GLM76544	2002	Germany, Sachsen-Anhalt	2166	–	NT
<i>B. picridis</i>	<i>P. hieracioides</i>	GLM75203	2002	Germany, Sachsen-Anhalt	2176	NT	ND500
<i>B. picridis</i>	<i>P. hieracioides</i>	GLM65715	2004	Germany, Sachsen-Anhalt	2170	–	NT
<i>B. picridis</i>	<i>P. hieracioides</i>	GLM75689	2005	Germany, Sachsen-Anhalt	2165	NT	36.1605 ^g
<i>B. saussureae</i>	<i>Hemistepta lyrata</i>	KUS-F22301	2006	China, Yantai	2510	–	ND500
<i>B. saussureae</i>	<i>H. lyrata</i>	KUS-F19842	2003	South Korea, Chunchon	2511	–	NT
<i>B. saussureae</i>	<i>H. lyrata</i>	KUS-F19951	2003	South Korea, Namyangju	2512	–	NT
<i>B. saussureae</i>	<i>H. lyrata</i>	KUS-F20026	2003	South Korea, Namyangju	2513	–	ND500
<i>B. sonchicola</i>	<i>Sonchus oleraceus</i>	221	2015	Greece	3329	NT	35.8529 ^g
<i>B. sonchicola</i>	<i>S. oleraceus</i>	KUS-F18997	2002	South Korea, Jeju	2515	–	NT
<i>B. sonchicola</i>	<i>S. oleraceus</i>	JK-F0944	2015	Spain	3328	NT	35.7525 ^g
<i>B. sonchicola</i>	<i>S. oleraceus</i>	KUS-F19364	2002	South Korea, Jeju	2514	–	NT
<i>B. sp.</i>	<i>Jurinea alata</i>	GLM74350	2003	Germany, Sachsen-Anhalt	2116	–	NT
<i>B. sp.</i>	<i>C. sonchifolia</i>	KUS-F19490	2003	South Korea, Chunchon	2509	–	NT
<i>B. sp.</i>	<i>C. sonchifolia</i>	KUS-F19257	2002	South Korea, Hongcheon	2508	–	NT
<i>B. taraxaci</i>	<i>Taraxacum officinale</i>	GLM75104	1997	Germany, Sachsen-Anhalt	2250	–	NT
<i>B. taraxaci</i>	<i>T. officinale</i>	GLM73772	2001	Germany, Sachsen-Anhalt	2252	–	NT
<i>B. tulasnei</i>	<i>Senecio vernalis</i>	GLM73530	2002	Germany, Sachsen	2186	–	NT
<i>B. tulasnei</i>	<i>S. vernalis</i>	GLM76784	2002	Germany, Sachsen	2187	–	NT
<i>B. tulasnei</i>	<i>S. vernalis</i>	286	2015	Greece	3326	NT	ND500

initial denaturation followed by 55 cycles of 95°C for 10 s and 56°C for 30 s. The nomenclature for interpreting all qPCR results were followed as described by Bustin et al. (2009).

Exogenous IC. To identify whether there was any alteration in amplification efficiency due to the presence of PCR inhibitors, an IC as described by (Haudenshield and Hartman 2011) was used with the *B. lactucae*-specific primers tailed on, so that only a single primer pair was needed to amplify *B. lactucae* DNA, and the IC target (Supplementary Fig. S1B). The core sequence was designed to hybridize to a 22-base oligonucleotide probe (BI_IC_Probe). The 5' end of the probe was labeled with Quasar 670 and the 3' end with Black Hole Quencher-3 (Biosearch Technologies, Inc.). The melting temperature of this probe was similar to that of the BI_probe and, when amplified by F17 and R18, generated an amplicon the same size as the *B. lactucae* target. All oligonucleotides were purified with polyacrylamide gel electrophoresis (IDT). A 100 attoMole working stock was prepared in EDTA for use as an IC. To the above qPCR master mix, 8 aM of IC was used as a target for each reaction, equivalent to 60 copies. A standard curve prepared using DNA extracted from different sporangial counts (BI-Gr assay) was used as the template in reactions to determine whether the IC would interfere with the accuracy of the BI-Gr assay (BI-Gr-IC assay).

Cloning of PCR products, sequencing, and sequence analysis. Selected qPCR products were purified using Agencourt AMPure XP beads (Beckman Coulter), cloned into pCR4.0-TOPO (Invitrogen), and sequenced in both directions with T7 and T3 universal primers (Eton BioSciences). Multiple sequence alignments were performed using Clustal W (Hall 1999).

Sporangia volume measurements. To assess the variation in sporangia volume, images of 30 different sporangia were captured on a compound microscope (Olympus model BX60F; Olympus Optical Co. Ltd.) using a $\times 40$ objective. Using a hemocytometer, the diameter of the sporangia was measured using ImageJ software (ImageJ 1.49v; National Institutes of Health). Because sporangia are nearly spherical, the radius was used to calculate the approximate volume of the sporangia.

Data analysis. Of the 24 replicates for a sporangial count of 1 determined by flow cytometry, *B. lactucae* DNA was not detected in 5 of them and 1 replicate that had a C_q value of 30; these 6 replicates were considered outliers and, therefore, removed from the analysis. Similarly, 1 of the 24 replicates for a sporangial count of 5 had an outlier with a C_q value of 30 and was removed from the analysis. Also, for sporangial counts of 10, 15, 40, and 50, outliers having a C_q value difference of >1.5 within the same dilutions were excluded from the analysis. For the other counts of sporangia determined by flow cytometry used to generate the standard curve, 12 replicates of each were included. Amplification of DNA from the sporangial dilution series was also examined under the same conditions as those in which field samples would be processed to evaluate whether amplification inhibitors made it through the DNA extraction process. The approach was the same as described by Klosterman et al. (2014), with the exception that there were four randomly picked replicates for each sporangial count. Efficiency of qPCR was calculated using the formula $[-1 + 10^{(-1/\text{slope})}] \times 100$. Linear regression was run on the sporangial numbers and the corresponding C_q values using the ggplot2 package in RStudio (version 0.99.467; RStudio, Inc.). The standard curve graphs also were generated in RStudio.

RESULTS

Sequence analyses for TaqMan assay probe and primer development. Comparative genomic analysis of the mtDNA sequences using alignments of 69 different mitochondrial genomes of *B. lactucae* in addition to related oomycetes enabled identification of unique primer annealing sites that were used to amplify mtDNA from *B. lactucae* but did not amplify mtDNA of closely

related species; this was similar to an approach used by Bilodeau et al. (2014) for *Phytophthora* spp. The assay used herein enabled detection of DNA from all 35 *B. lactucae* isolates recovered from a diverse temporal and worldwide geographic distribution (Table 1).

Assay specificity, efficiency, and limit of detection. Assay specificity was tested by both conventional PCR (Fig. 1) and qPCR (Table 1). Conventional PCR was performed on 71 isolates representing 13 different *Bremia* spp., which were collected from 18 different hosts from different geographic locations (Table 1). Of the 71 isolates of *Bremia* spp. tested, only 34 isolates of *B. lactucae* from two hosts (*L. serriola* and *L. sativa*) amplified with a product size of 96 bp (Fig. 1A and B). Similarly, to test the specificity, the TaqMan assay was deployed using the BI_probe and primers (F17 and R18) on template DNA from 21 different isolates representing 11 different *Bremia* spp. collected from various geographic locations (Table 1). Of the 21 isolates tested, 15 isolates were not detected, despite using 500 or 5 pg of target DNA template. Specific template DNA amplification from the positive control isolates of *B. lactucae* (C14C1452 and C14C1492) yielded a C_q value of 28.71 and 29.95, respectively, when 2 pg of target DNA was used in the reaction. The nucleotide sequence of the positive control was identical to the *B. lactucae* sequence used to design the probe. DNA from the remaining four isolates (2144, 2165, 3328, and 3329) was detectable but with a lower signal ($C_q > 35$). The quantity of DNA used for these four TaqMan assays to amplify DNA template were either 5 pg (2144) or not quantifiable (2165, 3329, and 3328) (Table 1). As expected, there was no detection signal observed in any negative controls in each plate.

The amplification of the BI-DNA assay exhibited a linear response until the DNA concentration was <20 fg, with an amplification efficiency of 103% (Supplementary Fig. S2). Given the high sensitivity of detection, C_q values > 35 were considered nonspecific for this TaqMan assay for field application.

Correlation between C_q values and sporangial counts. There was a significant linear relationship between sporangial counts (isolate C14C1452) and C_q using DNA extracted from 14 different sporangial counts, ranging from 1 to 1,000 sporangia (BI assay) (Fig. 2; Supplementary Fig. S3). The amplification of the BI assay exhibited a linear response, with an $R^2 = 0.927$. The average C_q values of the majority of the sporangial counts were well within the 95% confidence interval, except for 40, 50, and 100 sporangia (Fig. 2A).

Impaction spore samplers were placed in a pathogen-free field for 3 days. Sample rods were then processed for DNA extraction as described below and an aliquot was added to purified sporangial DNA prior to amplification. The BI assay was not negatively affected by the addition of sample rod DNA extract (efficiency of 98.44%). The C_q values of *B. lactucae* in the presence of sample extract (BI-Gr assay) were reduced by an average of 0.4 when compared with BI assay. For each count examined by qPCR, no significant differences between the standard curves (with and without sample rod extract) were observed (data not shown). However, there was a decrease in the slope, by 0.31, indicating that sample rod extract enhanced amplification efficiency from 88 to 98% (Supplementary Table S1; Fig. 2). The C_q values of 32.23, 29.24, and 22.80 correspond to 1, 25, and 1,000 sporangia, respectively (Fig. 2B). The majority of the mean C_q values fall within the 95% confidence interval, except for 30, 40, 50, and 100 sporangia (Fig. 2B). The regression presented in Figure 2B was used to calculate the approximate number of sporangia for all sample rods from field experiments.

Analyses of amplification efficiency of the BI-Gr assay in the presence of the IC. In the presence of the IC target and BI_IC Probe and primers, the reaction rate of *B. lactucae* detection was decreased by a factor of 6.5 (the y-intercept increased by 2.14 cycles), although slope and linearity were maintained (Fig. 3; Supplementary Table S2). For example, there was an increase in the C_q value for 25 sporangia of *B. lactucae* when the IC was added

($C_q = 29.24$ without the IC versus 30.83 with the IC). However, the target for the IC was present in low amounts (60 copies/reaction) and was expected to yield C_q values of approximately 32 to 33 in the negative reaction (reaction mixture with IC target and not *B. lactucae* target DNA). Increase in the C_q values for the IC at these dilution levels was also observed in amplifications with increasing sporangial counts (Fig. 3). In this assay, the C_q values of the IC varied significantly or were not detected when the C_q values of *B. lactucae* were <29 (Fig. 3). For example, IC was not detected when the sporangial counts were >300 .

The reaction rate of the BI-IC assay (BI assay + IC) was decreased by a factor of 7.1 (the y-intercept increased by 2.35 cycles), although slope and linearity were maintained with a correlation of $R^2 = 0.829$ (Supplementary Fig. S4). Aside from those values associated with counts of 5, 40, 50, and 100 *B. lactucae* sporangia, the average C_q values of most of the sporangial counts were well within the 95% confidence interval.

Detection and quantification of downy mildew inoculum from spore traps. The *B. lactucae*-specific primers and probe were evaluated with 75 spore trap samples obtained from several

Fig. 1. Amplification of the target locus from different *Bremia* spp. recovered from multiple plant hosts and geographic locations. **A**, Different *Bremia* spp. are highlighted in various color. The red arrow shows the expected product size of 96 bp. **B**, Marker amplified the DNA from different isolates of *Bremia lactucae* recovered from different parts of the world. Superscripts a, b, and c indicate California, Arizona, and international isolates, respectively. No product was observed in water control.

different field sites in the Salinas Valley (King City, Castroville, and USDA-ARS, Salinas) (Fig. 4A). Of the 75 spore trap samples from King City and Castroville, 24 and 23 samples, respectively, tested positive for *B. lactucae* during the sampling periods shown (Fig. 4C and D). The majority of the samples had C_q values < 30.00 (Fig. 4C), although the non-DNA template control samples prepared with grease-coated impaction spore traps and extracted for DNA were negative for amplification (data not shown). The average C_q value was relatively high (33.18 ± 2.02 standard deviation [SD]) during the months of July through mid-August. In contrast, the average C_q value from mid-August through mid-September was 28.64 ± 2.11 SD, equivalent to approximately 80 sporangia calculated from the standard curve (Figs. 2B and 4D). This C_q value corresponded with the onset of disease that was observed in early September (data not shown).

The effect of the IC concentration on the results of the spore trap samples. As the concentration of the *B. lactucae* target DNA increased, competition for the primers between the pathogen template and the IC led to an increase in C_q value for the IC (Fig. 5, left-hand side of the red line). For example, all the samples from 1 to 30 with C_q values < 29 for detection of *B. lactucae* showed a very high C_q value or no detection of the IC (Fig. 5). There were five samples (sample numbers 71 to 75) whose *B. lactucae*-associated

C_q values were above the level of sensitivity ($C_q > 35$) in the absence of IC, which could be due to variations in the number of mitochondria to sporangia. Though volume of sporangia may not always correspond directly to increases of mitochondria in the cell, there were size variations recorded among sporangia examined (Supplementary Fig. S5). Likewise, increasing the concentration of the IC resulted in a rise in the C_q value when *B. lactucae* DNA was present in moderate amounts (200 fg) (data not shown). The presence of the IC in this assay had only a limited effect on pathogen amplification (for the IC at 60 copies/reaction, there was a slight increase in the C_q for pathogen detection). A C_q of 39.3 (sample 6) was observed for the IC when present at 60 copies/reaction; in other spore trap samples with high inoculum densities, IC was not detected (samples 1 to 11, except 6) (Fig. 5). There was no detection of *B. lactucae* in two samples with and without IC (samples 76 and 77), like that in the water control (sample 78).

Multiple sequence alignment of qPCR products. Although no signs of downy mildew or symptoms were observed at the Castroville site in the period of July to August 2015, pathogen DNA was detectable with a C_q value of 34.92 ± 3.36 SD, such as DNA from the sample collected on 29 July 2015. To confirm the presence of *B. lactucae* in these samples with relatively higher C_q values, the qPCR products of this sample, including two positive controls (20

Fig. 2. Fourteen-point standard curves quantifying *Bremia lactucae* target amplification from different sporangial counts. **A**, Standard curve based on TaqMan assay (BI assay) amplification of purified DNA from 1 to 1,000 sporangial count. All data points are from an average of 9 to 23 biological replicates. See Materials and Methods for details. **B**, Standard curve based on TaqMan assay amplification of purified DNA in the presence of extract from impaction spore traps from 1 to 1,000 sporangial count (BI-Gr assay). In both A and B, the log number of sporangia is plotted against the quantification cycle (C_q) values. All data points are from an average of three technical replicates. The center line represents the line of fit and the gray area around the line is the 95% confidence interval. Error bars represents standard deviation.

Fig. 3. Relative frequency of target amplification of *Bremia lactucae* from impactation spore traps at three locations in the Salinas Valley, CA. **A**, Map showing spore trap locations, within circles: green, 2015-King City; dark blue, 2015-Castroville; red (2013-14 USDA); and light blue (2014-15 USDA). **B**, Dots indicate the relative frequency of *B. lactucae* amplification from spore traps at USDA (red and light blue dots) locations; **C**, at King City (green dots); and **D**, at Castroville (dark blue dots). Error bars represent standard deviation from eight (2015-King City), and four (2015-Castroville, USDA 2013-14, and USDA 2014-15) spore traps.

Fig. 4. Standard curve for the quantification of *Bremia lactucae* in a multiplex TaqMan assay of purified DNA in the presence of DNA extract from impactation spore traps (black circles) and internal control (8 aM = 60 copies) (black triangles) from sporangia enumerated using cytometry (BI-Gr-IC assay). The log number of sporangia is plotted against the quantification cycle (C_q) values. All data points are from an average of four biological replicates each. Blue line represents the line of fit and gray line around the blue line is the 95% confidence interval. Error bars represents standard deviation.

sporangia + grease and 20 sporangia) with a C_q value of 29.23 ± 0.41 SD, and 29.63 ± 0.15 SD, respectively, were sequenced by cloning into TOPO vectors, revealing the 86 bp of DNA sequence identical to that of wild-type *B. lactucae* in all samples, including the positive controls.

Detection and quantification of downy mildew inoculum during lettuce-free periods. In all, 28 trap samples were collected during a lettuce-free period (7 to 21 December). Even though lettuce is not commercially grown in Monterey, Santa Cruz, and San Benito Counties during this time (Van Bruggen and Scherm 1997), *B. lactucae* DNA from the 28 impaction traps was detectable in Monterey County (Fig. 4B). Of the 14 sample dates collected for the year 2013, qPCR analyses of those samples from four dates had a $C_q < 35$.

DISCUSSION

A TaqMan assay for the detection and quantification of *B. lactucae* from impaction trap sample rods was developed that could detect a single sporangium from field samples by targeting mitochondrial sequences that are in high copy number in sporangia. In addition, an amplification exogenous IC for the qPCR assay was developed to identify the potential effects of PCR inhibitors. It was possible to multiplex the assay in order to examine reaction efficiency and accuracy of quantification.

The TaqMan assay developed was designed for specific amplification of a unique locus in the mtDNA of *B. lactucae* and, in this regard, is somewhat different compared with other qPCR assays that have typically targeted the ITS or intergenic spacer of the rDNA, sequence-characterized amplified region, β -tubulin, or other nuclear genomic sequences (Bilodeau et al. 2007, 2014; Demontis et al. 2008; Klosterman et al. 2014; Martin et al. 2012; Schena et al.

2013; Schroeder et al. 2013). Because mitochondrial DNA is present in a higher copy number than most nuclear DNA loci, the unique species-specific mtDNA locus used in this study as the target increased the sensitivity of the assay compared with low-copy target nuclear sequences (Caldwell et al. 2015).

The qPCR assay developed for *B. lactucae* shows enhanced detection sensitivity compared with the previous genus-specific PCR assay developed for *B. lactucae* targeting the ITS region (Mota et al. 2011). Specificity is also enhanced due to reported variation in copy number of the rDNA repeat as observed in several eumycotic fungi and a few oomycetes (Bilodeau et al. 2012; Martin 2009; Spies et al. 2011). Analysis of standard curves using different sporangia concentrations indicate that even one sporangium could be accurately quantified using the assay with a C_q value of 32.23 when assayed under conditions similar to field evaluations. The standard curve constructed using DNA isolated from pure *B. lactucae* indicated that as little as 20 fg of pathogen DNA could be accurately quantified using the assay with a C_q value of 35.23. This value represents the practical limit of the assay because qPCR on several DNA samples from two other *Bremia* spp. yielded C_q values > 35 (see below); as a result, we note that C_q values ≤ 35 are classified as specific to *B. lactucae* and applicable to field evaluations.

Twenty-one isolates of *Bremia* spp. were tested using a TaqMan assay. Of the 21 isolates, 15 were not detected using the species-specific primers and probe. Of the remaining six isolates, two *B. lactucae* isolates (C14C1452 and C14C1492) were detected with a C_q of 29.95 and 28.71, respectively, with 2 pg of DNA each whereas, the last four isolates, representing single representatives of *B. lapsanae* and *B. picridis* and two isolates of *B. sonchicola* (2144, 2165, 3329, and 3328, respectively), were detected with $C_q > 35$ with ≤ 5 pg of DNA; for some of these isolates, only one of three

Fig. 5. Effect of internal control (IC) in measuring the relative frequency of *Bremia lactucae* target amplification from spore traps in both singleplex and multiplex reactions. The mean quantification cycle (C_q) values of target DNA (*B. lactucae*) and IC in a multiplex reaction are represented by orange and green dots, respectively, whereas blue dots represent mean C_q values of same sample DNA in a singleplex reaction, with no IC. The pink box shows the expected range of C_q values for IC, without interfering with the detection of target DNA. The red line indicates the threshold of *B. lactucae* target DNA, below which the detection of IC is variable. Error bars represent the standard deviation of three technical replicates.

technical replicates showed a signal. It is possible that this signal could be due to contamination and, given the extreme sensitivity of the assay, due care must be exercised while setting up the reactions.

The IC was used to prevent false-negative results and evaluate the presence of PCR inhibitors that may alter amplification efficiency of the target DNA. The IC was designed so that it used the same amplification primers as *B. lactucae* to further simplify running the assay. Depending on the concentration of the IC added to the master mix and the inoculum density of *B. lactucae* that was present in the sample, some competition in the amplification of the IC or pathogen target was observed (Bilodeau et al. 2012). The IC increased the C_q values of *B. lactucae* on both the standard curve (sporangial counts) and also on the field samples (spore traps); however, the samples with highest C_q values (lowest signal) were still linear. Hence, when running the assays with the IC, and the pathogen C_q is >30, it is best to first confirm that PCR inhibitors are not influencing the amplification efficiency (C_q for the IC in these wells is the same as the IC control) and then rerun the amplification without adding the IC to get an accurate estimation of the pathogen.

The results of this current work indicate that *B. lactucae* can be readily detected in air samples when coupled with impaction spore trap systems like those deployed in earlier studies to detect *Peronospora* spp. (Klosterman et al. 2014). The qPCR analyses revealed that the field samples collected from the King City north site had an average $C_q < 30$. However, the C_q value of the spore traps located approximately 4 km to the south had a C_q value of 24.28 (data not shown). The low C_q value obtained from these spore traps correlates with the direction of the wind. In the Salinas Valley, winds are predominately from the northwest in months spanning the sampling period of April through July, with increasing offshore flows during winter months (CIMIS 2015). Because the wind is predominantly sweeping from north to south across the ranch, the average C_q values obtained from qPCR analysis of the north and south were 29 and 24, respectively, presumably because the wind deposited more *B. lactucae* on the southern traps that arose from the lettuce crops that were intervening between the north and south trap sites.

In contrast, in the months of August to September, spore trap samples were collected in Castroville near a field that contained no known downy mildew infection. The higher C_q values obtained from the initial qPCR analyses of the spore trap samples in July and August may have been associated with increased temperatures during this period (CIMIS 2015). At later stages of the crop near harvest, we observed downy mildew but disease incidence was low. Thus, the sensitivity assay enabled detection below the levels we could detect using visual scouting. Similarly, the assay enabled detection of the pathogen in spore samples during lettuce-free periods in both 2013 and 2014. These results suggest that the pathogen is present in cultivated lettuce grown in home gardens or on wild species of lettuce (Van Bruggen and Scherm 1997) that may persist in the Salinas Valley. In 2014, there was precipitation and cloud cover during December (CIMIS 2015) that was favorable to inoculum production or disease development (Su et al. 2004).

The described TaqMan assay provides an accurate and rapid means for quantification of *B. lactucae* over a broad range of inoculum densities in agricultural fields. The ability to accurately quantify small inoculum levels of *B. lactucae* sets the stage for a monitoring system using spore traps for sporangia detection, which will help lettuce growers determine the level of downy mildew threat and time fungicide applications in their fields, as seen the in system for *P. cubensis* (Gent et al. 2009; Granke et al. 2014; Hausbeck 2014). In addition to helping growers with effective timing of fungicide applications, the species-specific marker for *B. lactucae* can be useful in phenomics by detecting the pathogen in asymptomatic plant tissue.

The TaqMan assay developed herein enabled *B. lactucae* detection in the field at several sites in central coastal California,

and we have begun to assess the practical application of the assay to recommend fungicide sprays at a particular ranch. To further assess the relationship between inoculum density and disease development, we are examining the correlation of spore sampler data from multiple locations within the Salinas Valley, CA with disease severity weather data and existing forecasting models. Multiplexing the assay for simultaneous detection of both *B. lactucae* and *P. effusa* is underway. Meanwhile, combining this assay with the use of commercial DNA extraction kits, automated DNA extraction techniques, and a 384-well format for processing samples will facilitate high-throughput analysis for faster turn-around. The qPCR TaqMan assay provides the potential for an additional tool to integrate into an IPM strategy to manage lettuce downy mildew and minimize economic loss due to this disease.

ACKNOWLEDGMENTS

We acknowledge funding from the California Leafy Greens Research Program and the California Department of Food and Agriculture (grant SCB14043). We thank B. McLaughlin and J. E. Van Dyke for expertise and guidance in sorting sporangia using the BD influx instrument at the University of California-Davis Flow Cytometry Shared Resources; R. Pena, L. Ochoa, and F. Wong (USDA-ARS, Salinas, CA) for collecting spore trap samples; A. I. Putman for insightful review of the manuscript; and B. Smith for help in data analysis. The mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

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