Measurements of Aerial Spore Load by qPCR Facilitates Lettuce Downy Mildew Risk Advisement

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Abstract

The lettuce downy mildew pathogen, *Bremia lactucae*, is an obligate oomycete that causes extensive produce losses. Initial chlorotic symptoms that severely reduce the market value of the produce are followed by the appearance of white, downy sporulation on the abaxial side of the leaves. These spores become airborne and disseminate the pathogen. Controlling lettuce downy mildew has relied on repeated fungicide applications to prevent outbreaks. However, in addition to direct economic costs, heterogeneity and rapid adaptation of this pathogen to repeatedly applied fungicides has led to the development of fungicide-insensitivity in the pathogen. We deployed a quantitative PCR assay-based detection method using a species-specific DNA target for *B. lactucae* coupled with a spore trap system to measure airborne *B. lactucae* spore loads within three commercial fields that each contained experimental

plots, designated EXP1 to EXP3. Based upon these measurements, when the spore load in the air reached a critical level (8,548 sporangia per m³ air), we advised whether or not to apply fungicides on a weekly basis within EXP1 to EXP3. This approach saved three sprays in EXP1, and one spray each in EXP2 and EXP3 without a significant increase in disease incidence. The reduction in fungicide applications to manage downy mildew can decrease lettuce production costs while slowing the development of fungicide resistance in *B. lactucae* by eliminating unnecessary fungicide applications.

Keywords: oomycetes, vegetables, disease management, cultural and biological practices, epidemiology, disease warning systems, pathogen detection

Downy mildew is an important foliar disease of lettuce (*Lactuca sativa* L.) worldwide and is caused by *Bremia lactucae* Regel, an obligate oomycete phytopathogen (Choi et al. 2011; Raid and Sui 2012; Wu et al. 2017). Lettuce is the leading vegetable crop in California with a farm gate value of \$2.4 billion. Monterey County alone accounts for more than 60% of lettuce production in California (California Agriculture Statistics Review 2017-2018). In lettuce, the disease causes light green to yellow angular spots on the leaf, along with a white, fluffy, downy (downy mat like) growth on the abaxial surface. Since downy mildew is primarily a foliar disease, these leaf symptoms reduce the marketability of lettuce and may lead to further losses during transit and storage (Wu et al. 2017). Until recently, resistant cultivars, fungicide application, and cultural practices were adequate to manage outbreaks of the disease in endemic areas

(Kunjeti et al. 2016). Novel variation of *B. lactucae* has led to the emergence of numerous races over the past two decades, and thus plant breeding efforts have not been able to keep pace with the emerging pathotypes (Kunjeti et al. 2016). Thus, fungicides have remained an effective option against this pathogen to significantly reduce crop losses (Koike et al. 2017; Raid and Datnoff 1989). However, emergence of fungicide-resistant races of the pathogen (Brown et al. 2004; Crute et al. 1987; Raid and Sui 2012; Raid and Datnoff 1990; Schettini et al. 1991), and the loss of registration of many fungicides due to concerns over environmental safety and adverse impact on human health, have limited the available fungicides for the management of lettuce downy mildew (Brown et al. 2004; Crute et al. 1987; Wicks et al. 1994). Without the means to adequately control disease, there are also food safety concerns associated with downy mildew infections on lettuce, as these infections and subsequent lesions can lead to secondary infections of nonphytopathogenic enteric pathogens such as *Escherichia coli* O157:H7 (EcO157) and *Salmonella enterica* Typhimurium (*S. Typhimurium*) (Simko et al. 2015).

The necessity for effective and alternative management practices has led to the use of currently approved fungicides before infection as a means of reducing disease outbreaks (Koike et al. 2017; Zijlstra et al. 2011) and is a practice currently favored by growers. In such a scenario, application of fungicides, even before the development of the disease, is as effective way to manage the disease in field or greenhouse conditions. Most available fungicides for this disease management strategy are protective rather than curative in nature (Koike et al. 2017), and thus sometimes 4 to 6 fungicide applications are applied beginning 1 or 2 weeks after thinning to prevent an outbreak of the disease on a single crop. Such overuse of chemicals is not consistent with the current regulatory objectives to reduce their usage and prevent the development of fungicide insensitivity in the pathogen populations (Raid and Sui 2012). Development of fungicide insensitivity in *B. lactucae* has been well documented for the phenylamide fungicide metalaxyl and fosetyl-Al in California and Florida (Brown et al. 2004; Crute et al. 1987; Raid and Datnoff

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1990; Schettini et al. 1991). Fungicides with different modes of action, assigned group numbers by the Fungicide Resistance Action Committee (FRAC), are rotated to prevent fungicide resistance development. There are at least nine different FRAC codes registered for the management of lettuce downy mildew in California, and some examples of those in use by the industry include Reason (Reason 500 SC, fenamidone, FRAC 11, Bayer), Manzate (mancozeb, FRAC M3, UPI), and Revus (mandipropamid, FRAC 40, Syngenta) (Koike et al. 2017).

Downy mildew of lettuce is a polycyclic disease that is caused by successful infections from airborne sporangia (Fall et al. 2016) that in turn result in a second generation of sporangia in as few as 8 days (Fall et al. 2015). Hence, the chances of a disease outbreak over the growing season increases not only from downy mildew incidence from within the field but also from the inoculum from adjacent lettuce fields. Although frequent applications of fungicides have proven effective in preventing epidemics, they could also be unnecessary and escalate the cost of production.

Spore trapping and other early detection techniques for quantification of fungal and oomycete pathogens hold promise in forecasting disease outbreaks (Carisse et al. 2009; Gent et al. 2009; Granke et al. 2014; Mahaffee and Stoll 2016; Schena et al. 2013; Úrbez-Torres et al. 2010; West and Kimber 2015; West et al. 2008; Zijlstra et al. 2011). Previous work in our laboratory enabled quantification of the fluctuations in the amount of *B. lactucae*, further suggesting that it may be feasible to deploy the airborne detection of *B. lactucae* (Kunjeti et al. 2016) to formulate a spore trap-based downy mildew risk advisory to optimize the timing and number of fungicide applications necessary for disease control. Coupled with the weather data such as those that coincide with optimal temperature for infection between 4 and 15.5°C for *B. lactucae* (Su et al. 2004), an inoculum detection system could prove effective in reducing fungicide applications and delay the emergence of fungicide resistance in *B. lactucae*. Reductions in fungicide applications in turn could translate into lower production costs to the growers.

The objectives of this study were to determine whether disease risk levels based on quantitative PCR of *B. lactucae* from spore impaction samplers on a local scale could be used to effectively time fungicide applications for disease control, and secondly to determine which, if any, weather parameters are associated with increases or decreases in the detectable DNA from the airborne sporangia. This work represents an initial proof of concept for using spore impaction samplers for the quantification of airborne sporangia of *B. lactucae* for disease predictive purposes to reduce fungicide applications during a cropping cycle.

Materials and Methods

Experimental field plots and placement of impaction spore trap sampler rods. Three different experimental plots, designated as EXP1 to EXP3, were established within three different commercial lettuce fields near Salinas, CA, during April to October in 2016. The field for EXP1 was planted 31 March 2016 and harvested 9 June 2016; the field for EXP2 was planted 2 June 2016 and harvested 5 August 2016; EXP3 was planted 27 July 2016 and harvested 3 October 2016. Romaine type lettuce cultivar ‘Green Towers’ was used in all three plantings following standard commercial practices with 25 to 30.5 cm between plants (Smith et al. 2011). The dimensions of each experimental plot within the fields were 6.1 × 36.6 m (Fig. 1). Each treatment and the untreated control were replicated four times, and each treatment area was subdivided into sections of 2.0 × 9.1 m (w × l) (Fig. 1). The three treatments were replicated and randomized, and the treatments included: 1) no spray, 2) calendar-based spray applied about weekly, and 3) the spore-trap advised spray. The commercial field surrounding each EXP1 to EXP3 was sprayed with fungicides for control of downy mildew on a schedule determined by the grower. For impaction spore trap sample collection, two impaction spore traps were employed. One spore trap was placed on the north side, and the second trap was placed on the south side of each of the

lettuce fields (Fig. 1) since the predominant wind direction in the Salinas Valley is either from the northwest or southeast (Choudhury et al. 2017).

Impaction spore trap sample collection, DNA extraction, and quantitative PCR. Flow cytometry-based *B. lactucae* sporangial counts and Cq values using DNA extracted from sporangia were highly correlated (Kunjeti et al. 2016). Employing this method, we sampled *B. lactucae* sporangia in the air over lettuce fields using impaction spore traps and sampler rods as described previously (Klosterman et al. 2014). The type of impaction spore traps used in this study are the same as those used previously for detection of *B. lactucae* (Kunjeti et al. 2016), and placed so that the sampling arms were approximately 53 cm above the soil. Samples from two impaction spore traps were collected twice per week, on Mondays and Thursdays, over a period of 8 to 10 weeks for EXP1 to EXP3. DNA was extracted as described in Kunjeti et al. (2016).

The primers and probe used for the study were previously evaluated for species-specificity and characterized for use in qPCR (Kunjeti et al. 2016). The species-specific primers and probes were designed to amplify an 86-bp region close to the 5′ end of a unique 861-bp putative open reading frame (orf286) of unknown function in *B. lactucae* that was not present in other oomycete taxa. Briefly, a 5′ flap (lowercase letters) was added to the primers; F17 (5′-aataaatcataaGTCATTGTTTGATTAACT-3′) and R18 (5′-aataaatcataaGAGCTAGATTACCA-3′); BL_probe, 5′-ATCAATAGAATGTCCCACTGCAAT-3′ (Kunjeti et al. 2016). The 5′ end of the probe was labeled with FAM (fluorescein), and the 3′ end with Black Hole Quencher-1 (BHQ1; Biosearch Technologies, Inc., Novato, CA). Additional *in silico* analyses since

Fig. 1. Design and dimension of the experimental plots for impaction spore trap sampling for the lettuce downy mildew pathogen, *Bremia lactucae*, in field plot experiments within commercial romaine lettuce fields near Salinas, CA in 2016. Three beds (36.58 m × 2.02 m) were set up side by side, and the treatments were randomized for no spray (control), regular/calendar spray, and spore trap advisory spray. Each of the beds was subdivided into four plots of 9.14 m × 2.02 m for the above purpose. Additional details on sprays deployed and buffer zones are described in the methods. Two solar powered impaction spore traps were set up at the either end of the field and were sampled twice every week, for the entire period of cultivation, for the detection of *B. lactucae*.

the original publication (Kunjetti et al. 2016) revealed that this sequence of nucleotides is still unique to *B. lactucae* and is not similar to any other sequence in the GenBank database.

All qPCR experiments for determining the Cq values that corresponded to a previously calculated spore count (Kunjetti et al. 2016) were performed in 384 well plates using a LightCycler 480 II real time PCR machine (Roche Diagnostics, Basel, Switzerland). The qPCR reactions were run in triplicate using one DNA extraction per spore trap. The qPCR reaction volume of 12 µl contained 200 nM probe, 200 nM of each primer, 1× real master mix (5 PRIME, Hilden, Germany), and 1 µl of DNA extracted from the impactation spore trap rods. The LightCycler 480 software (release 1.5.0) and the Absolute Quant/fit Point analysis were used to run all of the qPCR assays for *B. lactucae* DNA quantification with a reaction profile of 10 min at 95°C initial denaturation followed by 55 cycles of 95°C for 10 s and 56°C for 30 s.

Fungicide application based on spore trap advisory. Preventative fungicides Manzate (Manzate Flowable, mancozeb, UPI, PA, U.S.A.), Reason (Reason 500 SC, fenamidone, Bayer, NC, U.S.A.), and Revus (mandipropamid, Syngenta, CA, U.S.A.) were applied in succession at a rate of 0.95 kg, 0.23 liter, and 0.23 liter, respectively, in a total spray volume of 542.51 liters/ha with the surfactant Syl-tac at a final concentration of 0.05% (vol/vol) manually using a CO₂ backpack sprayer set at 2.41 Bar, a 4-nozzle hand-held spray boom (47.5 cm spacing), and Teejet 8005 flat fan nozzles. Materials were applied in the equivalent of approximately 608 liters/ha. For the plots designated for calendar spray, the first two calendar sprays were done with Manzate followed by Reason and Revus for the third and final sprays, respectively (Table 1). For the plots designated for spore trap-based advisory sprays, only Revus was applied. The application schedule is provided in the supplementary section/data (Table 1).

The spore trap-based advisory to determine whether or not to apply fungicides was based on a Cq value of 24 from the qPCR analyses of the spore trap sample coinciding with the first detection of plants with downy mildew symptoms. Based on extensive replicated analyses of standard curves using known amounts of sporangia of *B. lactucae* (Kunjetti et al. 2016), we were able to interpolate the approximate levels of airborne sporangia collected within the 72- or 96-h sample collection intervals. The volume of air sampled by each spore trap (V) = rod area (m^2) × number of rods (n) × rotating arm diameter (D) × 3.14 × RPM × duration of sampling in minutes (T) as determined earlier (Thiessen et al. 2016). Thus, (V) = (0.0012 × 0.04) m^2 × (1) × (0.085 m) × (2,400 rpm) × 3.1416 × (4,320 min) = 132.89 m^3 . This means that the threshold Cq value of 24 corresponding to 1,136 spores equates to 8.548 sporangia per m^3 air.

Collection of disease incidence data. Every plant within each experimental area was visually examined each week for downy mildew symptoms and sporulation. Plants within plots were counted as positive for disease when there was one or more symptomatic leaf. There were approximately 600 romaine lettuce plants per treatment area, as full lettuce plants are planted with spacing of about 25 to 30.5 cm apart (Smith et al. 2011). The entire plot was 9.14 m long, but on each end we did not evaluate plants in the last of the

0.76 to 0.91 m, which represented the buffer zone of each treatment area within each plot. So, the evaluation part of each plot is about 7.62 m long with approximately 0.76 m of buffer on each end (9.14 m total length).

Collection of weather data. Weather data for all experimental sites EXP1 to EXP3 were generated by Fox Weather, LLC using the MtnRT custom software described by Fox (2011). For the basic initial data, we used the North American Regional Reanalysis (NARR), obtained from NOAA at 32 km grid spacing and 3-h time step. From the NARR data, we produced sequential nested WRF runs (4 km grid) to provide input for MtnRT. Using MtnRT, running at a 1.5 km grid, we produced a continuous record of hourly data, including temperature, relative humidity, leaf wetness, and wind direction and speed at 2 m above the ground. The location coordinates for the weather data calculation, within 100 m of each experimental plot, were 36° 68' 96" N, 121° 59' 09" W at an elevation of 45 m.

The MtnRT processing included the interpolation to 1-h increments for the surface weather data, including wind direction and speed (Mesinger et al. 2006). This included consideration of MtnRT's calculation of wind vector variations resulting from development of nocturnal inversions. Wind speed was calculated at 2 m height above the ground instead of the standard 10 m height. This was done to better represent wind speeds more closely approximating the canopy level for lettuce. The calculation of wind speed at 2 m was based on a logarithmic relationship of wind speed versus distance above ground. A simplified version of the theoretical logarithmic relationship is $U_2 = U_1 (\ln(h_2/z_0)/\ln(h_1/z_0))$, where U_2 is wind speed to be calculated (in this case at $h_2 = 2$ m), while U_1 is the speed at the reference height (h_1). The value of roughness length (z_0) for most purposes would be 0.1 to 0.2 for the experimental location. Implementation for near-surface wind profiles in MtnRT provides inputs to operational forecasts in many different types of terrain and roughness conditions. For this project, we used the standard logarithmic wind profile in our operational version of MtnRT. All wind calculations are referenced above the plant canopy.

When necessary, supplemental weather data were collected from the nearby Salinas CIMIS weather station (Salinas South II, Station #214) and the Salinas Municipal Airport weather station (KSNS). Weather data collected from the CIMIS station included hourly measurements of temperature, relative humidity, wind speed, and wind direction. The association of weather parameters relative to increases or decreases in detectable levels of *B. lactucae* DNA was examined for intervals between 12:00 and 6:00 A.M. and 6:00 to 10:00 A.M. Sporangia are produced during periods of high humidity and lower wind speed, conditions prevalent during the hours of 12:00 to 6:00 A.M. during the lettuce growing seasons of the Salinas Valley, while increases in wind speed and temperature during the 6:00 to 10:00 A.M. interval are critical for dispersal of *B. lactucae* (Wu et al. 2001).

Statistical analyses. The area under the disease progress curve (AUDPC) was calculated based on days after planting (dap). Disease incidence was calculated as a percentage of the number of plants with downy mildew symptoms among the total number of plants examined in each replication. Analyses of variance were conducted using SAS (version 9.4) on the incidence data from EXP3, and treatments were compared with Fisher's protected least significant difference ($P < 0.05$).

Correlation analyses were conducted using R with data from EXP1 and EXP3 to determine the possible relationship between weather variables such as temperature, wind speed, and relative humidity and the levels of detectable DNA from the airborne sporangia (i.e., the Cq values from the qPCR).

Results

The Cq values collected across the three experiments by qPCR (Fig. 2) were used to determine the approximate corresponding number of sporangia for all sample rods from the field experiments. In the 72 to 96 h spore trap sample collection intervals, the corresponding

Table 1. Schedule of fungicide application on lettuce to manage downy mildew caused by *Bremia lactucae* based on calendar spray or advisory spray, and untreated control during the third field experiment (EXP3)

Untreated	Regular calendar spray schedule ^a	Spore-trap advisory spray schedule
No Spray	22 Aug. 2016 Manzate	No Spray
No Spray	2 Sept. 2016 Manzate	No Spray
No Spray	9 Sept. 2016 Reason	No Spray
No Spray	19 Sept. 2016 Revus	19 Sept. 2016 Revus
	Harvested – 3 Oct. 2016	

^a Fungicides Manzate, Reason, or Revus were applied on the dates listed with a backpack sprayer as described in the methods.

sporangia counts per impaction spore trap ranged from 7 sporangia (0.052 sporangia/m³ air) detected on 28 April 2016 ($C_q = 31.99 \pm 0.47$), to a peak of 605,306 sporangia detected (4,554.94 sporangia/m³ air) on 19 May 2016 ($C_q = 13.91 \pm 0.1$). The average C_q values on these dates from the two impaction spore traps placed on the north and south ends of the field were 30.88 (0.13 sporangia/m³) on 28 April 2016 and 14.86 (2,967.50 sporangia/

m³ air) on 19 May 2016 (Table 2; Fig. 2). To further minimize variation arising from changes in local wind speed or wind direction, the average from two spore traps set up at either end of the field was taken. In May 2016, we observed downy mildew symptoms and sporulation on three plants in the field surrounding EXP1. Detection of *B. lactucae* DNA at the nearby impaction spore trap samplers corresponded to the qPCR-based average C_q value of

Fig. 2. The average quantification cycle (C_q) values from quantitative PCR from DNA isolated from two solar powered impaction spore traps using a *Bremia lactucae*-specific primer and probe set previously deployed (Kunjeti et al. 2016) for detection of the lettuce downy mildew pathogen, *B. lactucae*. The traps were placed at the opposite end of the experimental lettuce plot for all three field trials shown in chronological order in (A), (B), and (C). Spore traps were sampled twice per week.

23.3. Subsequently, fungicide applications to the field were advised when the Cq value obtained from the impaction spore trap samplers fell below the threshold of 24 (~1,136 sporangia equivalents).

For both EXP1 and EXP3, we observed correlations between changes in wind speed and/or temperature with corresponding changes in detectable levels of *B. lactucae* DNA. This is illustrated in Figures 3 and 4, where the detection interval preceding the two lowest Cq values (between the hashed red lines) is also marked by sharp increases in wind speed and temperature. A similar trend was observed during EXP3 (Figs. 5 and 6), where the two lowest Cq values were associated with increases in temperature and wind speed over the detection interval (between the hashed red lines). This also was evidenced by significant negative associations between temperature and Cq values during the third experiment at both intervals of 12:00 to 6:00 A.M. ($r = -0.41$, $P > 0.05$) and 6:00 to 10:00 A.M. ($r = -0.37$, $P > 0.05$). In EXP1, temperature was positively correlated with Cq values ($r = 0.25$) during these detection intervals. Wind speed and Cq values were positively correlated during both experiments 1 ($r = 0.25$) and 3 ($r = 0.52$ to 0.78) over the indicated detection intervals. Between relative humidity and Cq values, positive correlations were detected during EXP3 ($r = 0.29$ to 0.34). During EXP1, the correlation between relative humidity and Cq values was weak ($r = 0.04$, $P > 0.05$). In EXP2, a visual association was not observed between the two lowest Cq values and changes in wind speed and temperature (Figs. 7 and 8). As there were fewer data points for Cq values in EXP2, statistical analysis was not feasible.

For all experiments (EXP1 to EXP3), an average qPCR threshold Cq of 24 (corresponding to 1,136 spores) was used to advise fungicide sprays. That is, when the average level of *B. lactucae* DNA detection from the impaction spore traps was recorded with a Cq value of ≤ 24 , fungicide was applied in EXP3 (Fig. 1). For comparative purposes, fungicides were applied roughly once per week on regular calendar-based fungicide spray intervals (Fig. 1, treatment 2). Thus, relative to the calendar-based applications, spore trap-advised sprays enabled the savings of one fungicide application in EXP1 and EXP2, since the Cq values were recorded as ≤ 24 early in EXP1 and 2 (Table 2). For EXP3, however, Cq values of ≤ 24 were not recorded until about 1 month after the initial spore trap readings (Table 2), thus saving three fungicide sprays. No downy mildew was observed within experimental plots 1 and 2, although the increases in detectable *B. lactucae* DNA in these two experiments were nevertheless used in our in-house downy mildew risk advisory to advise fungicide application at Cq values ≤ 24 (Cq 24 = 8,548 sporangia per m³ air).

The records of disease incidence in EXP3 (Table 3) enabled calculations of the effectiveness of withholding fungicide applications (Table 1), relative to the levels of disease control whether regular calendar-based sprays were applied, or whether the spore trap advised sprays were made. The untreated replicates had the highest AUDPC of 387.3 in DAP unit as compared with 203.6 for calendar and 369.65 for advisory sprays (Table 3); disease incidence increased chronologically over the three different dates in September 2016. At the final observation date (28 September), 32.6% of the plants in the untreated replicates developed downy mildew, while 6.9% of the

Table 2. Average Cq values from the quantitative PCR from DNA isolated from the impaction spore traps over the course of all the three field trials (EXP1, EXP2, and EXP3) on the respective dates and the corresponding spore count per cubic meter of air over the test plots

Date	C _q Trap #1	C _q Trap #2	Average C _q	Spore count ^a	Spore count/m ³ air
EXP1					
4/22/16	25.12	28.04	26.58	306.52	2.31
4/25/16	23.12	25.22	24.17	1,174.91	8.84
4/28/16	31.99	29.78	30.88	17.67	0.13
5/2/16	25.48	27.31	26.39	277.4	1.57
5/5/16	28.79	24.42	26.61	435.77	3.28
5/9/16	23.3	19.59	21.45	9,333.61	52.68
5/12/16	-	24.88	24.88	615.35	4.63
5/16/16	25.91	27.28	26.6	228.45	1.29
5/19/16	13.91	15.81	14.86	394,350.57	2,967.50
5/23/16	15.53	18.23	16.88	129,377.92	730.16
5/26/16	18.2	20.71	19.46	24,635.23	185.38
6/2/16	22.48	22.45	22.47	2,798.9	10.54
6/6/16	19.52	21.86	20.69	10,954.1	61.82
6/8/16	21.28	25.24	23.26	3,183.64	23.96
EXP2					
7/4/16	-	28.71	28.71	55.27	0.42
7/6/16	25.05	26.18	25.62	385.34	2.90
7/11/16	25.61	-	25.61	387.77	2.92
7/14/16	24.29	30.67	27.48	452.51	3.41
7/25/16	20.07	24.86	22.47	6,614.84	49.78
7/28/16	19.64	23.5	21.57	8,990.49	67.65
8/1/16	16.93	22.91	19.92	46,417.06	261.96
EXP3					
8/25/16	29.11	27.89	28.5	67.75	0.51
8/29/16	26.5	26.97	26.74	193.3	1.09
9/1/16	28.16	27.99	28.08	82.49	0.62
9/6/16	28.87	-	28.87	49.98	0.38
9/8/16	25.65	25.93	25.79	347.63	2.62
9/12/16	24.87	28.99	26.93	331.86	1.87
9/15/16	26.3	25.82	26.06	295.57	2.22
9/19/16	23.94	23.8	23.87	1,158.53	6.54
9/22/16	27.04	25.3	26.17	314.52	2.37
9/26/16	23.78	23.34	23.56	1,419.83	8.01
9/29/16	22.13	23.81	22.97	2,328.26	17.52

^a Corresponding spore count calculated using formula as described in our previous work (Kunjeti et al. 2016). Columns with dashes represent loss of impaction spore sampler rods during the experiment.

plants in the regular calendar-based sprays had symptoms, and for the spore trap advised sprays 16% of the plants were symptomatic (Table 3). Downy mildew incidence between plots treated based on the calendar spray and spray advisory were not statistically significant ($P > 0.05$), but incidence in plots treated based on calendar spray was significantly ($P < 0.05$) lower than in the untreated plots.

Therefore, there was an average effective disease control of 79% in the regular calendar-based spray routine as compared with the untreated control replicates. There was nearly 50% disease control for the spore trap-based fungicide spray advisory on the final observation date (28 September), in which there were 104 diseased plants in the spray advisory group versus 211 in the untreated (Table 3).

Fig. 3. Average wind speed, temperature, and relative humidity for each day over a period of 6 weeks during the first field trial for impactation spore trap based advisory spray for lettuce downy mildew, caused by *Bremia lactucae*, in a romaine lettuce field plot (EXP1). Each point on the graph corresponds to a single date representing the average of five time points between a 6:00 A.M. and 10:00 A.M. block. The average quantification cycle (Cq) values were obtained by quantitative PCR from DNA isolated from two solar powered impactation spore traps using a *B. lactucae*-specific primer and probe set previously deployed (Kunjeti et al. 2016). The vertical dashed lines represent the two lowest Cq values from the spore trap sampling.

Discussion

The aims of this study were to assess whether impactation spore trap samplers could be deployed to time fungicide applications based on the load of detectable airborne *B. lactucae*, and to further assess environmental parameters that favor airborne detection and pathogen dispersal. Spore trap advised fungicide applications were made

based upon a numerical Cq value of 24 from qPCR cycles (corresponding to 1,136 spores, which equates to 8,548 sporangia per m³ of air) that was obtained in week 1 of EXP1, when sporulation was detected on a few plants on the north end of the field surrounding the experimental plot. In our previous work, we characterized a highly sensitive TaqMan assay for *B. lactucae* DNA derived impactation spore

Fig. 4. Average wind speed, temperature, and relative humidity for each day over a period of six weeks during the first field trial for impactation spore trap based advisory spray for lettuce downy mildew, caused by *Bremia lactucae*, in a romaine lettuce field plot (EXP1). Each point on the graph corresponds to a single date representing the average of seven time points between a 12:00 A.M. and 6:00 A.M. block. The environmental factors shown here for this block of time have been reported by various studies to be critical for infection of lettuce by *B. lactucae*. The average quantification cycle (Cq) values were obtained by quantitative PCR from DNA isolated from two solar powered impactation spore traps using a *B. lactucae*-specific primer and probe set previously deployed (Kunjeti et al. 2016). The vertical dashed lines represent the two lowest Cq values from the spore trap sampling.

trap samplers, and here we have applied the technique to develop a more efficient spray advisory for control of lettuce downy mildew.

In EXP1 and 2, no disease incidence was recorded within the experimental plots, although the increases in detectable *B. lactucae* DNA were nevertheless used in our in-house downy mildew risk advisory to advise fungicide application at Cq values >24 (equating to 8.548 sporangia per m³ air). Some variables in these

experiments set up in commercial fields were beyond our control, such as the extent of genetic resistance in fields planted with resistant lettuce cultivars. Thus, disease incidence may be reduced or absent if the pathotype population present in the area is unable to infect resistant lettuce genotypes. Different pathotypes of *B. lactucae* are known (Castoldi et al. 2012). Additionally, the weather parameters may not have been conducive for disease development

Fig. 5. Average wind speed, temperature, and relative humidity for each day during the third field trial for impactation spore trap based advisory spray for lettuce downy mildew, caused by *Bremia lactucae*, in an experimental field plot (EXP3) within a romaine lettuce field. Each point on the graph corresponds to a single date representing the average of five time points between a 6:00 A.M. and 10:00 A.M. block. The average quantification cycle (Cq) values were obtained by quantitative PCR from DNA isolated from two solar powered impactation spore traps using a *B. lactucae*-specific primer and probe set previously deployed (Kunjeti et al. 2016). The vertical dashed lines represent the two lowest Cq values from the spore trap sampling.

following periods of higher disease risk, even when sporangia counts were higher than the spray-threshold Cq value of 24.

Downy mildew incidence between plots treated based on the calendar spray and spray advisory were not statistically significant. However, there was a significant difference in disease incidence between the calendar-based spray treatment and the untreated control.

Fungicide application based on the spore trap-based spray advisory resulted in a savings of three sprays for EXP3 and reduced disease incidence by ~50% with respect to untreated plots. In EXP1 and EXP2, however, there was a reduction of one fungicide application per experiment.

Temperature, relative humidity, and wind speed have been shown to play a direct role on the sporulation mechanism in *B.*

Fig. 6. Average wind speed, temperature, and relative humidity for each day during the third field trial (EXP3) for impactation spore trap based advisory spray for lettuce downy mildew, caused by *Bremia lactucae*, within a romaine lettuce field. Each point on the graph corresponds to a single date representing the average of seven time points between a 12:00 A.M. and 6:00 A.M. block. The average quantification cycle (Cq) values were obtained by quantitative PCR from DNA isolated from two solar powered impactation spore traps using a *B. lactucae*-specific primer and probe set previously deployed (Kunjeti et al. 2016). The vertical dashed lines represent the two lowest Cq values from the spore trap sampling.

lactucae (Su et al. 2004, 2009). Optimal infection by *B. lactucae* occurs between 4 and 15.5°C along with an RH of at least 80% or more (Su et al. 2004). As can be seen from Supplementary Fig. S1, the weather conditions in the Salinas Valley correspond closely to the conditions required for *B. lactucae* infection to occur through much of the lettuce production season. Lettuce cultivation is limited to geographical regions with cool to moderate

temperatures, coastal fog, and high humidity, factors that also favor *B. lactucae* infection (Scherm et al. 1995). To this effect, we included these weather parameters in our study to determine if a relationship exists between these factors and the detectable pathogen DNA levels. Associations between weather data and Cq values in this study indicated a pattern that the period of increased detection of *B. lactucae* DNA at the impaction spore trap samplers was

Fig. 7. Average wind speed, temperature, and relative humidity for each day during the second field trial (EXP2) for impaction spore trap based advisory spray for lettuce downy mildew, caused by *Bremia lactucae*, in a romaine lettuce field. Each point on the graph corresponds to a single date representing the average of five time points between a 6:00 A.M. and 10:00 A.M. block. The average quantification cycle (Cq) values were obtained by quantitative PCR from DNA isolated from two solar powered impaction spore traps using a *B. lactucae*-specific primer and probe set previously deployed (Kunjeti et al. 2016). The vertical dashed lines represent the two lowest Cq values from the spore trap sampling.

marked by conditions that typically favor spore production and dispersal in *B. lactucae*. Specifically, peak periods of DNA detection, indicative of spore dispersal, were observed at higher levels in EXP1 and EXP3, in association with higher temperature, increased wind speed, and a drop in relative humidity between 6:00 and 10:00 A.M. *B. lactucae* spore dispersal significantly increases with decreasing relative humidity and increasing temperature during late morning

hours (Wu et al. 2001, 2005). The results of our study shed some light on the timing of dispersal, and further support those earlier findings with actual impactation spore trap data. However, further research using our methodology of impactation spore trap should analyze DNA from the airborne sporangia at multiple time points, sufficient enough to permit the establishment of the observed relationship between weather parameters and the detectable DNA amount.

Fig. 8. Average wind speed, temperature, and relative humidity for each day during the second field trial (EXP2) for impactation spore trap based advisory spray for lettuce downy mildew, caused by *Bremia lactucae*, in a lettuce field. Each point on the graph corresponds to a single date representing the average of seven time points between a 12:00 A.M. and 6:00 A.M. block. The average quantification cycle (Cq) values were obtained by quantitative PCR from DNA isolated from two solar powered impactation spore traps using a *B. lactucae*-specific primer and probe set previously deployed (Kunjeti et al. 2016). The vertical dashed lines represent the two lowest Cq values from the spore trap sampling.

Table 3. Mean downy mildew incidence (%) and associated standard errors of the mean (SE) on three dates in September 2016 in the third field experiment (EXP3) on lettuce and mean area under the disease progress curve following fungicide applications based on calendar spray, advisory spray, and the untreated control

Day of year	Downy mildew incidence (%) $\pm$ SE in		
	Untreated	Calendar based spray	Advisory spray
September 8, 2016	5.2 $\pm$ 0.78	3.7 $\pm$ 0.44	6.9 $\pm$ 0.39
September 21, 2016	12.5 $\pm$ 0.53	7.4 $\pm$ 1.10	11.7 $\pm$ 0.53
September 28, 2016	32.6 $\pm$ 5.87	6.9 $\pm$ 0.64	16.0 $\pm$ 3.86
Mean AUDPC	387.3	203.6	369.7

The fungicide applications in this study were advised based on the Cq values from the amount of DNA detected from spores captured by the rods. The fungicide applications were not based on combining advanced prediction of weather patterns. However, a close analysis of the relationships between the Cq values for detection of spores and weather parameters indicated a weather pattern reported in other studies that is favorable for production and dispersal of *B. lactucae* spores. Hence, we propose that the use of impaction spore traps for advanced detection in conjunction with advanced weather pattern prediction will provide an added layer of protection to crops and a simultaneous reduction in application of fungicides.

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