Project title:	Downy Mildew & Late Blight Control
Project number:	CP 184
Project leader:	Tim Pettitt, Eden Project Learning (EPL) Alison Lees, (JHI) Tom Wood, (NIAB)
Report:	Annual report, May 2021
Previous report:	Annual report January 2020
Key staff:	Alison Lees, Tom Wood, David Cooke, Jane Thomas, James Lynott, Simon McAdam, Anne Webb, Beatrice Corsi, Tim Pettitt.
Location of project:	EPL, Bodelva, Cornwall; JHI, Invergowrie Dundee; NIAB, Cambridge & RSK ADAS Boxworth, Cambridgeshire
Industry Representative:	Emma Garfield, R & D Agronomist and Head of Technical, G's Growers, Barway, Ely, Cambridgeshire
Date project commenced:	January, 2019

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

Despite the dry season resulting in relatively few downy mildew outbreaks combined with the effects of COVID 19 regulations restricting sampling opportunities, KE and laboratory access, some progress was made with typing of a limited number of *Bremia* isolates (3), initial knowledge transfer activities, and comparisons of molecular methodologies for testing of seed lots of basil and spinach. These tests showed that:

1) simply measuring levels of DNA present in seed and washings samples could lead to overestimates of viable pathogen levels;
2) despite this, measurements of either RNA levels or of DNA by qPCR after application of propidium monoazide (PMA) both showed promise in realistically estimating viable pathogen presence;
3) steam treatments of seed lots effectively reduced pathogen DNA by 50%.

Wherever possible, tasks not completed in 2020 have been rescheduled to 2021 (e.g. program of KT and the fungicide resistance testing of *Bremia* isolates).

Background

The oomycetes are a large group of fungus-like organisms many of which have evolved to become pathogens of plants. A large and varied group of oomycete plant pathogens are spread by air-borne and/or water-splashed propagules and cause diseases primarily of the above-ground parts of plants are collectively known as the Aerial Oomycetes. Horticulturally significant pathogens within this group are the **downy mildews** (main genera in horticultural crops: *Peronospora, Hyaloperonospora, Pseudoperonospora, Plasmopara, Bremia*), stem rots, shoot diebacks and blight caused by *Phytophthora* spp. as well as shoot and leaf 'blisters' caused by *Albugo* spp.

Diseases caused by aerial oomycetes typically exhibit rapid epidemics, which if left unchecked under optimal environmental conditions have the potential to cause complete crop loss either directly by mortality, or by rendering foliar and fruit produce unmarketable. Disease control options are limited or under-utilised and currently management is heavily reliant upon the use of fungicides, often used prophylactically, as none of the available chemicals can reliably achieve curative control, and once disease is observable in crops it will often already have become established and difficult to manage. Unfortunately, the number of currently available fungicides is becoming very restricted as a result of product withdrawals and too few new introductions. The resulting reduction in the number of active ingredients being used

in control programs greatly increases the risk of pathogen populations developing fungicide resistance. The use of resistant varieties, where available, is a good disease management option although their use puts huge selection pressure on oomycete pathogen populations for new races capable of overcoming host resistance. Cultural disease management methods (e.g. appropriate tilth management, removal/treatment of crop debris, manipulation of environmental conditions), often have a limited impact on disease when used alone but can greatly (even synergistically, e.g. control of {a non-oomycete with analogous epidemiology} *Botrytis* grey mould in ornamentals, O'Neill *et al*., 2002), increase the efficacy of chemicals and plant resistance in integrated management programs. Similarly, the use of rapid pathogen detection and disease simulation models can optimise the timing of fungicide applications and in some seasons reduce their number – increasing efficacy whilst reducing costs and potential environmental impacts.

The use of contaminated seeds is considered responsible for many outbreaks of downy mildews on basil and spinach, caused by *Peronospora belbahrii (Pb)* and *Peronospora effusa (Pe),* respectively. *Pe* is a seed-borne pathogen, producing heterothallic oospores in the seed coat (Kandel *et al.,* 2019) that cause systemic infection in the crop. The transmission of *Pb* on or in seed is less clear; only a single case of oospore production has been reported in basil (Cohen *et al.,* 2017) and most new disease outbreaks have been attributed to asexual aerial conidia and become evident only on relatively mature plants (Budge, Personal communication). *Pb* conidia have been observed in basil seed samples (Falach-Block *et al.,* 2019; Wood, 2021, personal communication) however, it is unlikely the propagules would remain viable for extended periods under unfavorable environmental conditions. Therefore, it is postulated that disease is propagated through mycelial infection inside the seed-coat (Jennings *et al.,* 2017).

Integrated pest and disease management (IPM) is an increasingly important and pertinent area of research for horticulture and this project aims, through the provision of reviews and best practice grower guides, to consolidate current knowledge, ensuring that measures that can be taken up are quickly disseminated and potential barriers to uptake identified. The project builds on current knowledge of several pathosystems (specifically downy mildew on lettuce, spinach and basil and to a lesser extent onion) to develop and validate the tools required for a long-term integrated approach to disease management. New tools for the genotypic analysis of *Bremia lactucae* populations, linked to phenotypic characteristics such as 'race' and fungicide sensitivity, will allow an understanding of population diversity to directly inform resistance deployment and breeding and fungicide stewardship to be greatly improved using an approach that has previously been highly successful for potato late blight (*Phytophthora infestans*, Ritchie *et al*., 2018). The other main strand of research focusses

on identifying/verifying primary inoculum in spinach and basil by detection and viability-testing of seedborne infection to steer future integrated management both by improved quality screening and providing effective tools for assessing cultural controls. Here we report on the second year's progress with limited KE, assessing pathogen races and progress with assessing molecular procedures for determining levels of viable downy mildew present in contaminated seed lots.

Summary

As stated above, a combination of a low incidence of downy mildew disease and the consequences of COVID 19 restrictions severely hampered progress with this project during 2020. Despite distributing a reasonably large number of mildew sampling packs to growers and consultants, only a limited number were sent to JHI as a consequence of the low incidence of disease outbreaks. This situation was further exacerbated by disruption to postal delivery services which resulted in delays and many samples degrading en route to a condition where it was not possible to recover viable isolates. Nevertheless some progress was made especially with seed testing work and the following tasks and findings were achieved:

- Three viable isolates of *Bremia lactucae* were obtained and typed using IBEB differential varieties and all appeared to closely resemble IBEB race BI:24EU. These isolates were stored for fungicide resistance testing in 2021.
- Molecular tests have been demonstrated to be capable of detecting and quantifying small amounts basil and spinach downy mildew (DNA/RNA) both externally and inside contaminated seed lots.
- Seed lots containing low levels of downy mildew (DNA/RNA) still potentially pose a high risk for growers
- Across the seed-lots tested a greater quantity of downy mildew DNA was detected inside the seed coat compared to seed washings.
- Steam-treated basil seed lots contained approximately 50% less downy mildew DNA than untreated samples from identical lots, indicating that steam-treatment reduces the pathogen load.
- RNA levels are generally considered to be more representative of the amount of viable pathogen present than DNA levels. In both washings and inside seed RNA levels were generally ten times less than DNA levels, showing that detection of pathogen

DNA levels can lead to over-estimation of the amount of potentially viable downy mildew. Nevertheless, RNA levels increased after steam-treatment relative to untreated seed, indicating a potential response in the pathogen to steam and that RNA may remain viable even when downy mildew has been neutralised. More work is needed to clarify this situation.

- Results of the work carried out on the project have been presented in five oral presentations at grower meetings and AHDB webinars.

Financial Benefits

Aerial oomycete infections significantly reduce crop yield, with those affecting plants in propagation, in particular, able to cause total crop loss, and those in ornamentals potentially causing the crop to become unmarketable (Wedgwood, *et al.,* 2016). Timely intelligence concerning prevalent phenotypes present in downy mildew populations has potential for significant financial benefits in terms both of managing fungicide resistance and the deployment of cultivars with suitable resistance genes. In addition, the detection and interception/treatment of infected seeds is likely to have a large impact on downy mildew incidence in crops such as basil, whilst the effective use of cultural controls and decision support systems could both reduce the frequency of spray applications, improve their efficacy and reduce the pressure selecting for new pathogen genotypes with fungicide resistance and/or capable of overcoming cultivar resistances.

Action Points

Action points are still not appropriate at this stage of the project.

SCIENCE SECTION

General Introduction

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Non-chemical seed-treatments using steam and hot water to neutralise pathogens whilst leaving the seedling viable have become more accessible to seed producers, enabling contaminated lots to be effectively sanitised (McGrath, 2019). Although seed de-sanitization approaches have been shown to be effective in reducing the effects of downy mildew in production settings, demonstrating that pathogens no longer remain viable in treated seeds is confounded using conventional DNA based-tools. Therefore, it is difficult to demonstrate the benefits to growers without them growing crops that may still be at risk from developing downy mildew. Provision of improved diagnostics with the capacity to distinguish the amount live pathogen in a seed lot could also be beneficial for the herb production sector to quantify the effectiveness of non-chemical seed treatments and help to reduce the incidence of new disease outbreaks.

Integrated pest and disease management (IPM) is an increasingly important and pertinent area of research for horticulture and this project aims, through the provision of reviews and best practice grower guides, to consolidate current knowledge, ensuring that measures that can be taken up are quickly disseminated and potential barriers to uptake identified. The project builds on current knowledge of several pathosystems (specifically downy mildew on lettuce, spinach and basil and to a lesser extent onion) to develop and validate the tools required for a long-term integrated approach to disease management. New tools for the genotypic analysis of *Bremia lactucae* populations, linked to phenotypic characteristics such as ‘race’ and fungicide sensitivity, will allow an understanding of population diversity to directly inform resistance deployment and breeding and fungicide stewardship to be greatly improved using an approach that has previously been highly successful for potato late blight (*Phytophthora infestans*, Ritchie *et al*., 2018). The other main strand of research focuses on identifying/verifying primary inoculum in spinach and basil by detection and viability-testing of seedborne infection to steer future integrated management both by improved quality screening and providing effective tools for assessing cultural controls. Here we report on the second year’s progress, assessing pathogen races in field crops and progress on using qPCR and RT-qPCR for detecting downy mildew infection and the determination of viable external

and internal seed-borne infections to establish if these techniques can accurately quantify the amount of living *Pb/Pe* present on or in a seed sample.

Materials and methods

Fungicidal control in IPM programmes:

Core Objective 6) Develop a service using validated tests to monitor fungicide sensitivity in key aerial oomycetes as listed. (WP3, O2 - links to consolidated programme of work on Bremia lactucae described in WP4).

Background

A range of different fungicide groups are currently available for the control of downy mildews in UK horticultural crops. However, some of the most important/effective fungicides currently deployed (e.g. azoxystrobin and metalaxyl M) are also highly prone to the development of fungicide resistance in their target pathogen populations. To some extent, these compounds have been 'protected' by the development of mixed formulations using multi-site active fungicides with anti-oomycete activity, such as mancozeb, to reduce the survival of resistant pathogen mutations. Nevertheless, the risk of resistance development remains high, hence the need for 'ringing the changes' with fungicide groups used in disease management programmes.

Collection of isolates

Industry stakeholders were contacted through personal contact, AHDB publications and newsletters and asked to submit disease samples for characterisation in the project. As a result of COVID-19 restrictions and nationwide lockdown it was not possible to request samples from Column stock growers. 65 sample packs and instructions (Figure 1) were posted to stakeholders for postage-paid return. Growers (lettuce, spinach, and tomato) were asked to sample 4 lesions per distinct outbreak wherever possible and to supply crop information. Additionally, an FTA® card was supplied onto which 4 different lesions could be sampled. This was to enable DNA from the outbreak to be stored (and analysed for genotypic characteristics) in addition to the live pathogen sample, or in cases where the live sample was not retrievable from the host material on receipt. The sampling strategy was aligned with that currently undertaken in the AHDB 'Fight against Blight' campaign with a view to a longer-term system for outbreak alerts or pathogen characterisation being compatible.

The acronym FTA stands for “Flinders Technology Association”. FTA CARDS are chemically treated Whatman filter papers that are designed for the collection, preservation and shipment of biological samples including clinical and environmental samples for subsequent DNA and RNA analysis. FTA cards are cotton-based, cellulose paper containing chemicals that lyse cells or biological samples, denatures proteins, and protects nucleic acids (which are immobilized onto the card’s matrix) from further destruction either by UV light or by oxidation or by nucleases. When cells or biological samples are applied to FTA cards the cells are lysed and the nucleic acids are immobilized and stabilized within the cards’ matrix. Apart from being used to collect biological samples, FTA cards can be used for storing or preservation of samples prior to their usage; and they can also be generally used to transport biological samples from one point to another before any further analysis such as DNA and RNA analysis can be performed on or with them. For example, FTA cards can be used to collect, preserve and transport biological samples from one continent or country to another. https://microbiologyclass.com/fta-flinders-technology-association-cards/

Lettuce Markers:

Core Objective 7) Provide a comprehensive package of work on lettuce downy mildew Bremia lactucae to include: development of markers with which to assess population diversity, association of race type, fungicide sensitivity and other phenotypic characteristics with genotype in anticipation of a service for provision of outbreak information to growers and to inform IPM and breeding activities.

Background: *Bremia lactucae* is an obligate, oomycete pathogen of lettuce that causes leaf chlorosis and necrosis and adversely affects marketability. The disease is managed with a combination of host resistance and fungicide applications. Fungicide applications are routinely made under the assumption that inoculum is always present during favourable environmental conditions. This approach often leads to fungicide resistance in *B. lactucae*

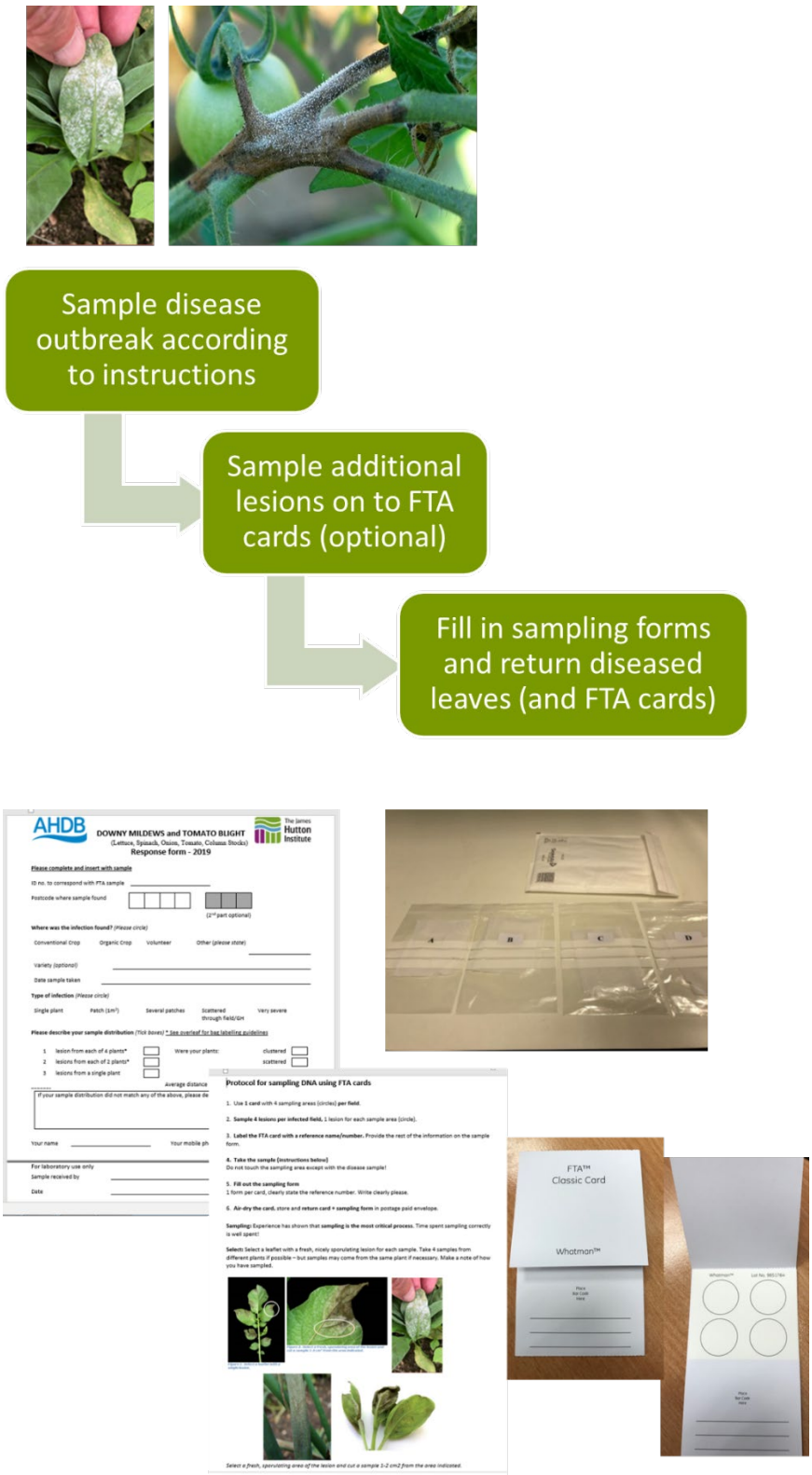

Figure 1. Sampling pack and instructions for oomycetes

populations. Similarly, the evolution of races of *B. lactucae* able to overcome host resistance in lettuce varieties threatens disease control and must be monitored to ensure effective use of existing varieties and breeding of new varieties. To this end, the International Bremia Evaluation Board (IBEB) is a joint initiative of lettuce breeding companies with a mission to identify new races of *B. lactucae* that pose a significant threat to the lettuce industry. A well-defined and internationally agreed system of race denomination (Parra et al. 2016) and a common set of host differentials and test protocols exist and will be adhered to in this project in collaboration with the IBEB.

SSR genotyping for *Bremia lactucae* (CP 186)

Work is currently underway to develop the SSR genotyping method for *B. lactucae* and is being carried out alongside this project by PhD student Alicia Farmer (project CP 186). Simple sequence repeats (SSR) were chosen to analyse the genomic diversity of the *B. lactucae* population, as they are an accepted way of discriminating genotypes based on length variation (alleles) at a range of points in the pathogen genome (loci). A combination of different markers with different allele lengths can create a unique genotypic profile for an isolate, essentially giving a 'fingerprint'. SSR markers are not diagnostic for specific traits but are a commonly utilised method to get a representation of population diversity. We hypothesise that isolates belonging to a common genotype profile will be more phenotypically similar than those with a different genotype. Therefore, we aim to explore associations between genotype and phenotype and factors including host cultivar, aggressiveness, fungicide sensitivity and location.

When samples of *B. lactucae* have been received for fungicide and race testing, DNA, spores and leaf material have also been stored and will be used in the marker development work. We have carried out race testing on the isolates obtained from 2020 outbreaks.

Race testing of *Bremia lactucae* isolates

B. lactucae isolates were assessed for race structure according to IBEB guidelines and protocols kindly supplied by Naktuinbouw in the Netherlands, who also supplied seed of the 16 current accessions in the official lettuce differential set (Set C). In brief, seed of each differential host (n=16) were germinated and grown on damp filter paper. Spores of each isolate maintained on lettuce seedlings were harvested into sterile distilled water. A sub-set of these spores were frozen for future use. Seedlings of each differential were inoculated with individual isolates of *B. lactucae* using a spore suspension containing 10 x 10^3 spores/ml.

Each set of differentials was incubated in a large box with a lid to maintain high humidity, kept in a glasshouse at 15°C under natural daylight conditions. Disease was assessed 12 days post inoculation using the IBEB scale as described below (Fig. 2).

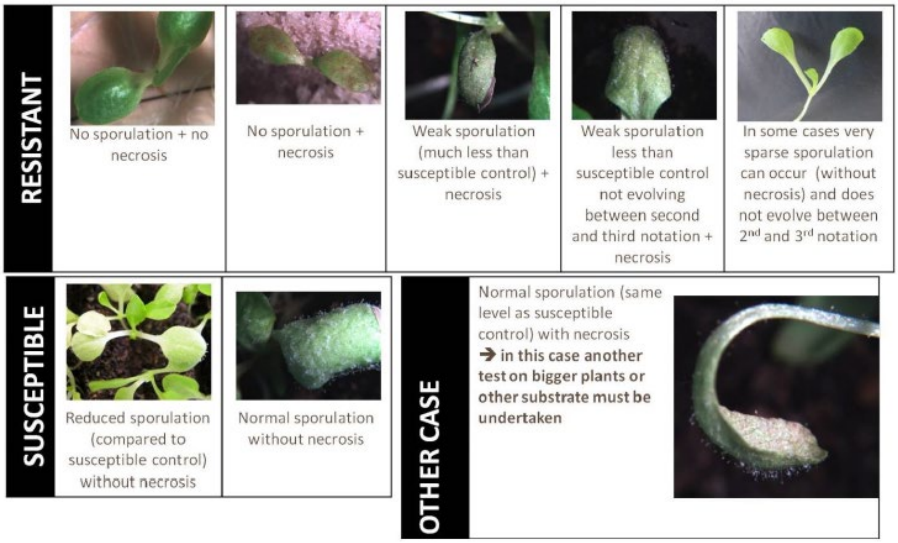

Figure 2. IBEB *Bremia lactucae* disease assessment scale.

Refining molecular diagnostic approaches to seed testing for viable downy mildew inoculum in sweet basil, spinach, and column stocks

Testing and validation of seed sampling procedures/WP 3 development of validated assays for provision of a commercial (seed) testing service for industry/stakeholders.

Comparison of the performance of qPCR and PMA-qPCR approaches for quantifying viable propagules in tissue and seed contaminated with *P. effusa* (*Pe*), *P. belbahrii* (*Pb*) and *H. parasitica/P. matthiolae*:

Background:

As the usage and range of products available for controlling downy mildew decreases, the primary strategies for maintaining effective control in basil and spinach are the use of clean, disease free seed and good sanitary practices in the growing environment. Obtaining clean seed is a primary concern for growers, yet sourcing pathogen-free seed can be a challenge, particularly where additional conventional grow-on screening is costly and often inconclusive in diagnosing downy mildew infection. In an effort to improve detection, DNA-based molecular diagnostics have been developed for both *Pb* and *Pe* and are capable of detecting even minute quantities of pathogen DNA (Shao & Tian, 2018; Feng *et al. 2014*). Unfortunately,

conventional PCR and quantitative PCR (qPCR) that detect DNA are limited in their ability to discern between live and dead sources, so estimating the level of risk posed to the grower using contaminated lots is extremely difficult.

An alternative strategy, Reverse Transcription quantitative PCR (RT-qPCR) can be utilized to establish the relative content of messenger RNA (Ribose Nucleic Acid) in a sample. mRNA is the intermediate molecule transcribed from DNA from which amino acids are translated into functional proteins in living cells. RNA is less stable than DNA and persists for only a very short time outside living cells. Therefore, accurate quantification of RNA levels can be used to estimate the amount of viable pathogen within a biological sample. This is achieved by converting the mRNA in the sample to cDNA (complementary DNA). Once the mRNA has been converted, the cDNA is then quantified using conventional qPCR apparatus, providing an estimation of the relative quantities of RNA present, and thus the amount of live pathogen.

The main aim of the work was to determine if established qPCR and RT-qPCR techniques could successfully be utilized to detect basil and spinach downy mildews on seed, and test how these perform in comparison to PMA-PCR (propidium monoazide PCR) viability approaches that are also under evaluation in the project (see CP 184 Yr 1 report - Pettitt, et al., 2020). PMA-PCR viability approaches have been demonstrated to be able to distinguish between the amount of live and dead *Pb* conidia however, photo-reactive PMA dye must physically permeate dead cells in order to function, so may have potentially limited application in detecting internal/mycelial contamination in hard seed-coats.

Quantification of spore suspensions:

Conidial suspensions of *Pb* and *Pe* (1 x 10^5/ml) for extracting DNA for generating the qPCR control standards were quantified in triplicate using a haemocytometer visualised on an Olympus BN2 microscope at 20 x magnification.

DNA extractions from conidia:

DNA extractions were performed using a DNeasy Plant mini kit (Qiagen, UK) according to the manufacturer's protocol; conidial suspensions (1 x 10^5/ml) were collected from the three respective downy mildew species in order to obtain template for quantifying the amount of DNA detected during qPCR testing. Spores were concentrated by centrifugation for 10,000 rpm for 60 secs before adding extraction buffer.

Seed washing and preparation:

Three x 100 seed samples of lots CP 1A-CP 8 (Table 3) were counted by hand and stored in individual 2.5 ml micro-centrifuge tubes. 1 ml of 70 % ethanol was transferred to each tube using a P1000 and clean pipette tip. Each sample was mixed vigorously for 60 secs on a

vortex. The effluent was then decanted into a clean micro-centrifuge tube. Any residual ethanol was removed with a clean pipette. Seed washings were concentrated by centrifugation for 10,000 rpm for 60 sec before the supernatant was removed. The ethanol-washed seed was dried overnight at room temperature before performing any DNA/RNA extractions.

DNA extraction from seed washings and seed:

Seed washings were homogenized in a genogrinder; extraction buffer was added and samples milled with 2 x 2.5 mm steel balls for 60 secs at 1000 rpm before extracting the DNA. Seed samples were ground in liquid nitrogen using a clean pestle and mortar. DNA extraction on basil seed and seed washings from basil and spinach were performed using a DNeasy Plant mini kit (Qiagen, UK), whereas a Wizard Food kit (Promega, UK) was used to extract DNA from Spinach. All DNA extractions were implemented according to the manufacturer's protocol.

RNA extraction from seed washings and seed:

RNA was extracted from prepared seed and seed washings using an RNeasy total RNA kit (Qiagen, UK) according to the manufacturer's protocol.

cDNA synthesis

Quantitative PCR/RT-QPCR:

Extracted DNA was analysed on an ABI Step one plus in a 96-well format using 10 µl volumes with triplicate technical replicates per sample. 5 µl SYBR green master mix, 1 µl of each F/R primer (0.5 µM), 1 µl H_20 and 1µl template DNA. Assays for detecting *P. belbahrii* (PbITS2-F: 5'- CTGAACAGGCGCTGATTG, PbITS2-R: GCAACAGCAAAGCCAATTC; *P. effusa:* PfsITSF: GTTCGATTCGCGGTATGATT, PfsITSR: TCACACAGCAAAGCCAATTC; were validated on pure DNA extracted from spores over a 10 ng-100 fg/µl dilution series. The temperature cycle for PCRs consisted of an initial denaturation (96°C) for 2 mins, followed by 40 amplification cycles alternating between 96°C for 15 sec and 60°C for 30 sec extension time per cycle. The number of cycles after which DNA could be detected against the baseline is termed the cycle threshold (Ct). The Ct is the number of cycles of PCR for the fluorescence signal generated during amplification to exceed a threshold. The greater the number of amplification cycles required to detect DNA indicated a lower starting quantity in the sample. In order to quantify RNA in seed samples it first must be converted into double stranded DNA for it to be detected by qPCR. This is achieved using a process called reverse transcription (RT), where recombinant enzymes are exploited to convert RNA molecules into complementary DNA' (cDNA). cDNA was prepared using a Maxima H Minus first strand

synthesis kit (Thermofisher, UK), using 1 µl of cDNA as template for qPCR reactions conducted as described.

Results

Fungicidal control in IPM programmes:

Lettuce (*Bremia Lactucae*):

Samples were received from 6 individual disease outbreaks (Bl1-6) in 2020. From these 6 outbreaks FTA samples were obtained from at least 1 lesion in 5 cases. It was not possible to obtain viable isolates from all samples received, this was due to delays in delivery resulting in the samples being received in poor condition. Three isolates from 2 individual outbreaks were successfully isolated from disease samples onto fresh leaf material. The sample material arrived too late in the season for these isolates to be effectively assessed for fungicide resistance, they were therefore kept for comparisons with isolates to be collected in 2021. The 2020 isolates were stored as spore suspensions and inoculated seedlings at -20°C and -80°C for future use.

Table 1: Isolates of *Bremia lactucae* obtained from 2020 outbreaks. Isolates with the same number and different letters originate from different samples within the same outbreak.

ID	County	Outbreak size	Date Collected	Date Received
2020_Bl2	West Sussex	Patch (1m2)	25/08/2020	28/08/2020
2020_Bl4E	Cambridgeshire	Several patches	16/09/2020	18/09/2020
2020_Bl4G	Cambridgeshire	Several patches	16/09/2020	18/09/2020

Column Stocks (*Peronospora parasitica*) and Tomato (*Phytophthora infestans*):

No isolates of *Peronospora parasitica* were received therefore fungicide tests could not be carried out.

No isolates of *P. infestans* were received from stakeholders in 2020 either within this project or through the AHDB Fight Against Blight campaign and fungicides tests could therefore not be carried out.

Spinach (*Peronospora effusa*):

Samples from 4 outbreaks in Spinach were received from different locations between July to October. However due to delays with delivery of samples the isolates were not in a condition suitable for maintaining on arrival therefore fungicide testing could not be carried out. FTA cards from each of these outbreaks have been retained.

Lettuce Markers:

Results of *B. lactucae* race tests:

Results of race testing of 3 isolates of *B. lactucae* are presented in Table 2. Two races were identified, which closely resembled a race designated by the IBEB committee (Bl24EU). There were no apparent problems with the tests; all of the universally susceptible control seedlings (cv. Green Towers) became infected with each isolate, providing confidence in the test method. Similar to the isolates tested in 2019, none were able to infect Dm3 (Dande) which appears to be a commonly overcome gene (from other available data). We will make a further analysis of this race data in comparison with all the publicly available data in the Bremia database to draw comparisons where possible.

Table 2. Race test results of 3 isolates obtained from 2020 outbreaks.

		Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille	C Sextet Code
			Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38								
Grid Position			**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	**S14**	**S15**	
Sextet Value			**1**	**2**	**4**	**8**	**16**	**32**	**1**	**2**	**4**	**8**	**16**	**32**	**1**	**2**	**4**	
Bl:24EU		+		+			+	+		+						(-)		50-02-00
2020_BL2	South	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	50-02-02
2020_BL4E	East	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	50-02-02
2020_BL4G	East	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	50-02-00

Refining molecular diagnostic approaches to seed testing for viable downy mildew inoculum in sweet basil, spinach, and column stocks

Comparison of the performance of qPCR and PMA-qPCR approaches for quantifying viable propagules in tissue and seed contaminated with P. effusa, P. belbahrii and H. parasitica/P. matthiolae:

Seed lots:

Ten seed lots were obtained for testing for DM contamination to date (Table 3). Basil lots CP 1 A and B, and CP 2 A and B comprised two commercial varieties with suspected *Pb* infection where batch A was left untreated and steam treatment was applied to batch B before the seed was sent to NIAB for testing. Lot CP 3 comprised home-saved cv Sweet Genovese seed collected from heavily infested plants (NIAB lot, PE 024, Ct 19.1), whereas CP 4 comprised a purportedly resistant cultivar that had previously tested positive for *P. belbahrii* (PE 024, Lot 21 Ct 31.1) by qPCR testing. Spinach lots CP 5-8 comprised four commercial cultivars suspected to be contaminated with *P. effusa.* The lots were sub-sampled in batches of 3,000 seed and divided into triplicate bulks of 100 seed for testing for the presence of *P. effusa/P. belbahrii* with the validated qPCR assays described in the 2020 report*. PCR testing of ethanol-washed seeds was implemented in 3 x 100 seed samples from each seed lot in order to assess for the presence of external contamination with downy mildew, followed by drying, homogenisation of the seed and extraction of DNA/RNA for performing the diagnostic tests. Conventional qPCR was used to confirm the presence or absence of *Pb* or *Pe* DNA in each of the 3 x 100 seed samples.

Table 3. Seed lots for testing in CP 184

Coded seed-lot	Species	Origin	Steam Treated
CP 1 A	Basil	CP 184	N
CP 1 B	Basil	CP 184	Y
CP 2 A	Basil	CP 184	N
CP 2 B	Basil	CP 184	Y
CP 3	Basil	(PE 024)	N
CP 4	Basil	(PE 024)	N
CP 5	Spinach	CP 184	N
CP 6	Spinach	CP 184	N
CP 7	Spinach	CP 184	N
CP 8	Spinach	CP 184	N

Table 4. Ct values and DNA content per µl washings (Wash.) and internal (int.) extracts from seed lots listed in Table 3 above. NB - Ct values for *Pb* and *Pe* DNA of the same concentration are not directly comparable due to differences in assay sensitivity.

Seed lot	Wash. Ct	Wash. DNA/µl	Int. Ct	Int. DNA/µl
CP 1 A	25.76	< 100 fg	23.01	< 1 pg
CP 1 B	25.37	< 100 fg	24.13	< 500 fg
CP 2 A	25.27	< 100 fg	22.53	< 1 pg
CP 2 B	25.73	< 100 fg	24.76	< 500 fg
CP 3	25.67	< 100 fg	22.60	< 1 pg
CP 4	26.35	< 100 fg	24.70	<500 fg
CP 5	25.95	< 1pg	29.24	~ 10 fg
CP 6	26.74	100 fg	25.81	< 1 pg
CP 7	28.07	<100 fg	23.48	>1 pg
CP 8	25.95	< 1 pg	0.00	Undetermined

Detection and quantification of *Peronospora belbahrii* DNA in Basil seed lots:

Quantitative PCR was used to assess the amount of total DNA (live and dead) extracted from each of the basil seed lots, and to assess if steam treatment had any reductive effective on *Pb* levels.

All six of the basil seed lots (CP1-CP4, Table 3) assessed tested positive externally and internally for *P. belbahrii (Pb)* DNA (Table 4 & Figure 3). DNA content in the external washings was quite similar across the six seed lots (< 100 fg/µl, Ct~25.5) and did not seem to be affected by steam treatment. *P. belbahrii* DNA contents inside the seed were observed to be approx. 5-10 times higher compared to the external levels. Internal *Pb* DNA levels ranged from <1 pg/ µl in CP 2A (Ct 22.53, untreated) and CP 3 (Ct 22.60, home-saved seed) to <500 fg/µl, (Ct~24.5) in the steam-treated samples CP 1B and 2B. Levels of DNA detected inside the untreated CP 1A (Ct 23.01) and CP 2A (Ct 22.53) samples respectively were observed to be approximately 50 % higher than inside seed from the steam-treated samples (CP 1B and CP 2B) of the same origin lot indicating that steam-treatment could have reduced *Pb* DNA content in CP 1B and 2B.

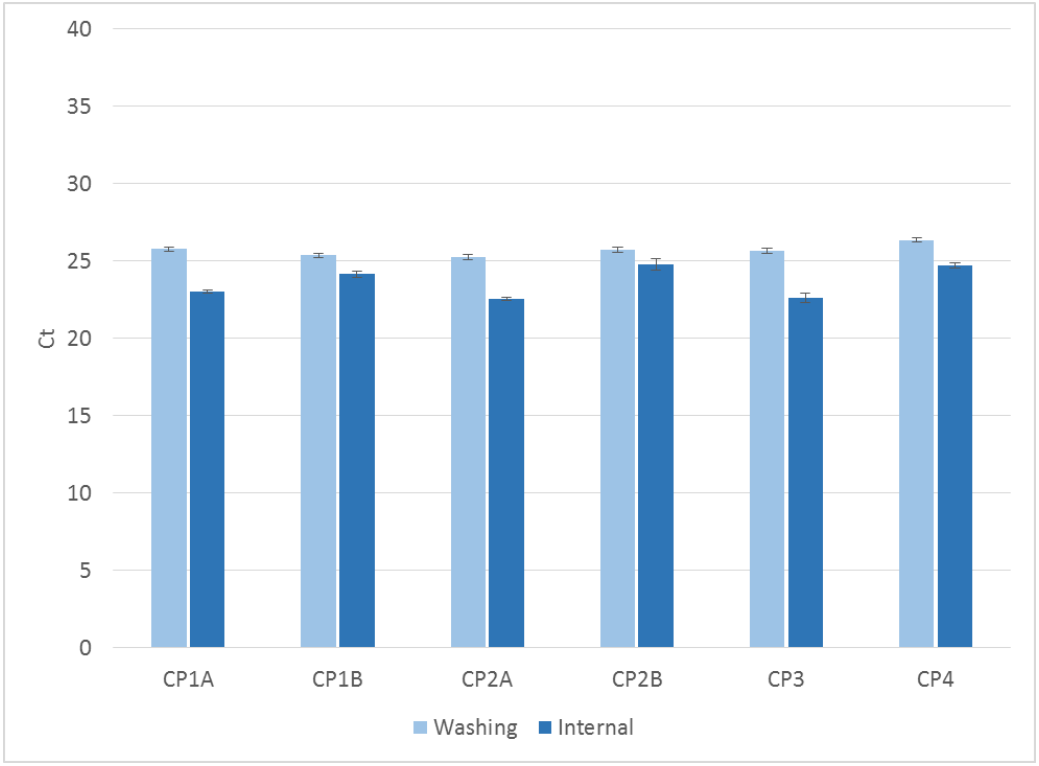

Figure 3. Histogram displaying Ct results from qPCR testing of DNA extracted from basil seed-washings and extracts from seed lots CP 1 A/B (untreated/steam treated, respectively), CP 2A/B (untreated/steam treated, respectively), CP 3 (home-saved Sweet Genovese seed), CP 4 (untreated commercial seed)

Detection and quantification of *P. effusa* DNA in Spinach seed lots:

Quantitative PCR was used to assess the amount of total DNA (live and dead) extracted from each of the spinach seed lots. All four spinach seed lots assessed tested positive for external contamination with *P. effusa.* (Table 4 and Figure 4). In contrast to basil seed lots, DNA content in the spinach external seed washings was more variable, ranging from < 1 pg/µl (Ct 25.95) in CP 5 and CP 8 to as low as < 100 fg/µl (Ct 28.07) in CP 7. DNA levels were higher externally in CP 5 compared to the amount of DNA detected inside the seed, with the opposite observed for the remaining three lots. Internal contamination of seed with *P. effusa* could only be confirmed in lots CP 5-7, with DNA contents ranging from ~10 fg/µl up to >1 pg/µl, respectively. No *Pe* DNA was detected inside seed from CP 8 (Ct undetermined), despite the extraction and testing being repeated twice. However, PCR was able to amplify the plant host DNA from all the seed samples indicating the extractions were of sufficient quality. Thus, the levels of pathogen may have potentially been below the threshold of detection.

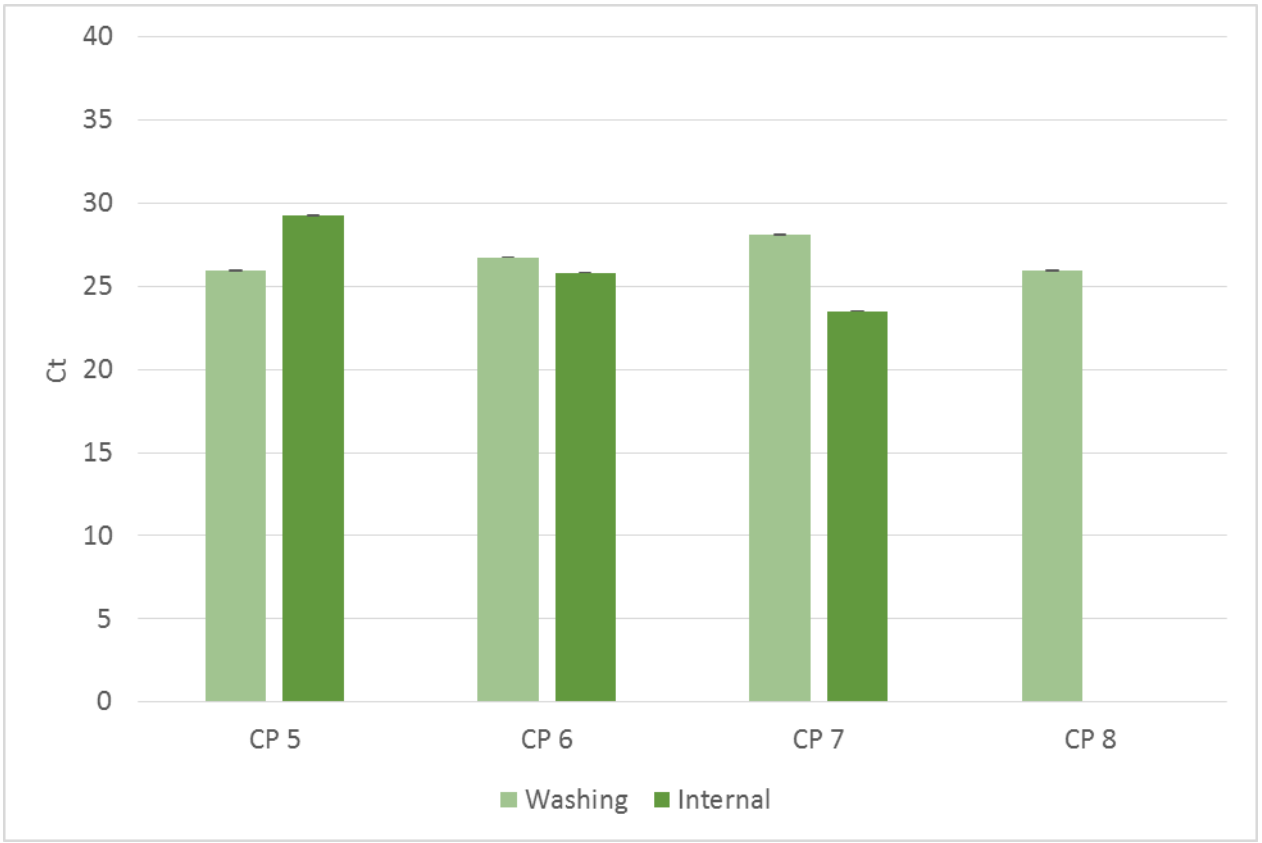

Figure 4. displaying Ct results from qPCR testing of DNA extracted from 3 x 100 spinach seed-washings and extracts from commercial seed lots CP 5- CP 8

Detection and quantification of *P. belbahrii* RNA (cDNA) in basil seed lots:

Quantitative PCR was used to assess the amount of RNA (cDNA) extracted from each of the basil seed lots, as a representative measurement of any live *Pb* present in the sample. Each of the six basil seed lots assessed tested positive externally and internally for *Pb* RNA (Table 5). The level of RNA detected in the external washings was comparable across the seed lots (< 10 fg/µl, Ct 29.9), with only CP 2A exhibiting lower content (< 1 fg/µl, Ct 32.02). The amount of RNA detected in external seed washings was on average ten times less compared to the amount of DNA detected in the same lots during the previous testing (Tables 4 and 5). In general, RNA levels were observed to be lower inside the basil seed than in washings and ranged from < 10 fg/µl to < 1 fg/µl. Lots CP 2B and 3 exhibited the highest levels of *Pb* RNA internally and this was similar to the levels observed in the washings.

Table 5. Ct values and RNA (cDNA) content per µl washings (Wash.) and internal (Int.) extracts from seed lots. NB - Ct values for *Pb* and *Pe* cDNA of the same concentration are not directly comparable due to differences in assay sensitivity.

Seed lot	Wash. Ct	Wash. cDNA/µl	Int. Ct	Int. cDNA/µl
CP 1A	29.97	<10 fg	33.24	< 1 fg
CP 1B	29.66	<10 fg	31.34	~ 1 fg
CP 2A	32.02	<1 fg	33.00	< 1fg
CP 2B	29.93	<10 fg	30.61	<10 fg
CP 3	28.68	~10 fg	29.41	<10 fg
CP 4	29.95	<10 fg	31.23	~1fg
CP 5	30.04	10 fg	35.16	<1 fg
CP 6	27.06	<100 fg	33.51	1 fg
CP 7	30.77	10 fg	34.71	<1 fg
CP 8	28.19	<100 fg	31.66	<10 fg

In general, the level of external and internal RNA *Pb* contrasted with the higher internal and lower external DNA levels observed in the same seed lots (Figures 3 and 5). RNA content inside basil seed was on average between 500-1000 times lower than the amount of DNA detected, apart from CP 3 where the concentration was closer to 100 times less. RNA content

was also observed to be lower internally in untreated lots CP 1A (<1 fg/µl, Ct 33.24) and CP 3 (~1 fg/µl, Ct 33.00), compared to heat-treated seed from the same respective lots with an approximate increase of six fold in CP 2 (~ 1 fg/µl) and ten-fold in CP 2B (10 fg/µl). This observation indicated that steam treatment increased pathogen RNA levels compared to the reductive effect in *Pb* DNA levels observed in the same lots.

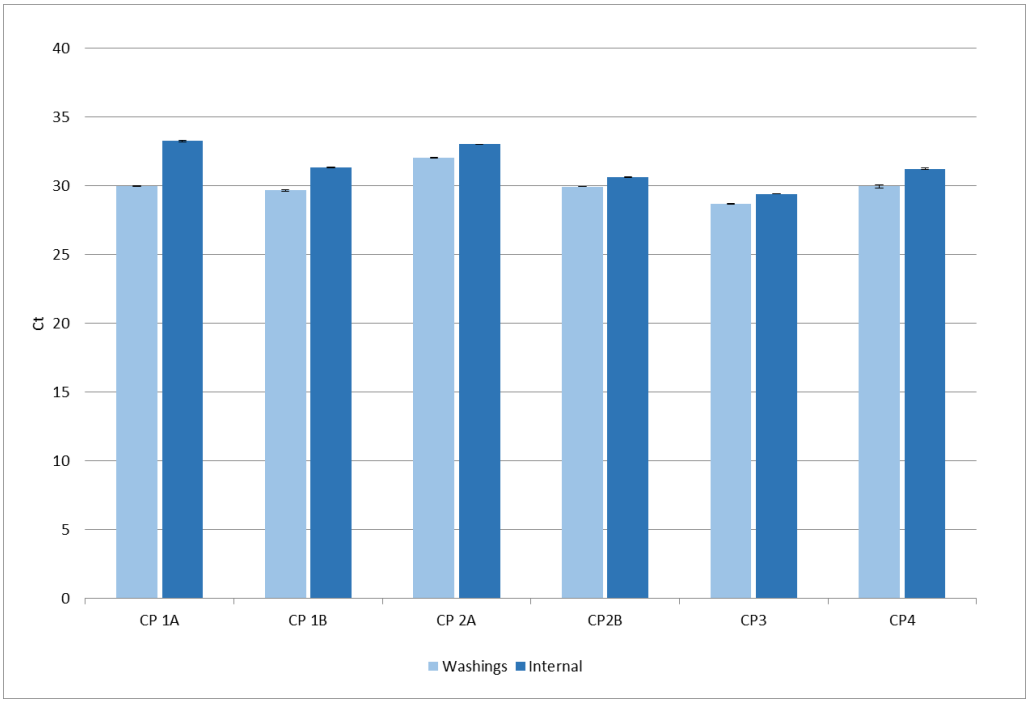

Figure 5. Histogram showing Ct results from RT-QPCR testing of RNA (cDNA) extracted from 3 x 100 basil seed-washings and extracts from seed lots CP 1 A/B (untreated/steam treated, respectively), CP 2A/B (untreated/steam treated, respectively), CP 3 (Home-saved Sweet Genovese seed), CP 4 (untreated commercial seed)).

Detection and quantification of *P. effusa* RNA (cDNA) in spinach seed lots

Quantitative PCR was used to assess the amount of RNA (cDNA) extracted from each of the spinach seed lots, as a representative measurement of any live *Pe* present in the sample. All four of the spinach seed lots assessed, tested positive externally and internally for *P. effusa* RNA (Table 5 and Figure 6). The level of RNA in the external washings was variable across the seed lots, ranging from approximately 10 to <100 fg/µl (Cts 30 - 27). The amount of RNA detected in the washings was approximately ten-fold less than the amount of DNA detected from the same lots. *Pe* RNA levels detected inside the seed were all consistently lower than those detected in the washings and ranged from <10 fg/µl (Ct 31.66, CP 8) down to <1 fg/µl (Ct 35.16, CP 5). The amount of *Pe* RNA detected inside the seed was greatly reduced compared to the amount of DNA detected in the same lots, with approximately ten times less

RNA in CP 5 and up to a thousand times less in CP 6 & 7. CP 8 was observed to have the highest internal RNA levels (Ct 31.66, <10 fg/µl) for the spinach seed samples even though the seed lot tested negative for *Pe* DNA.

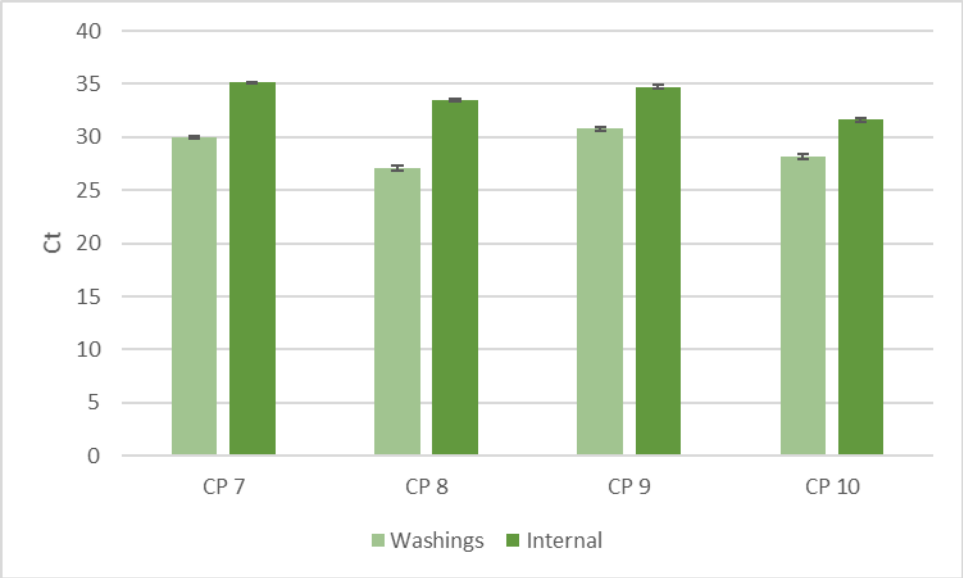

Figure 6. Histogram displaying Ct results from RT-QPCR testing of RNA (cDNA) extracted from 3 x 100 Spinach seed-washings and extracts from commercial seed lots CP 5-CP 8.

Discussion

Bremia race testing

Of the three isolates collected from discrete sampling locations during the 2020 growing season, one (2020_BL4G) matched the IBEB description BL:24EU with a sextet code 50-02-00, whilst the other two (2020_BL2; 2020_BL4E) were close matches with sextet code 50-02-02. This sample size is not sufficient to draw any substantive conclusions and the isolates have been stored for comparative fungicide resistance assessments together with the anticipated 2021 isolate collection.

Refining molecular diagnostic approaches to seed testing for viable downy mildew inoculum in sweet basil, spinach

The levels of external DNA contamination from *Pb* detected in basil seed washings were observed to be relatively uniform across seed lots (< 100 fg/µl), with ten times less than that detected inside the seed coat. Although external contamination has been reported previously in basil (Cohen *et al.,* 2017; Falach-bloch *et al.,* 2019), previous testing of seed washings did not identify any pathogen DNA (AHDB PE 024). The levels of internal contamination

appeared to be more variable than external levels, with some lots containing greater amounts of pathogen DNA than others. These differences indicated there could be variation in the amount of *Pb* colonizing the seed in the individual lots. CP 3 was observed to have the highest levels of internal *Pb* DNA compared to the other seed lots. This was anticipated as the seed was harvested from heavily infested plants. The qPCR testing also detected significant changes in DNA levels in steam-treated and untreated samples. Ct was increased by 1.12 cycles in the steam-treated lot CP 1B and 2.23 cycles in CP 2B compared to untreated seed in CP 1A and 2A, respectively. This was equivalent to a reduction of approximately 50% in the amount of DNA in the treated seed compared to the untreated seed, indicating that heat treatment reduced *Pb* DNA, which is potentially useful for pathogen control. However, subsequent testing of *Pb* RNA in the seed lots highlighted potential issues with over-estimation using the DNA-based approaches for quantifying downy mildew both on and in basil and spinach seed.

The levels of *Pe* DNA observed in spinach seed washings were generally more variable compared to *Pb* DNA in basil seed lots, although external DNA contents were observed to be similar in seed lots from both crops. *Pe* oospores are normally located in the testa (Kandel *et al*., 2019) so it was not surprising that levels may vary between the different spinach seed lots and different *Pe* isolates where levels of colonization may have varied. Internal levels of *Pe* DNA were lower and more variable than those observed for *Pb* in basil, indicating less *Pe* may colonize the seed compared to *Pb*. No DNA was detected inside seed from CP 8, despite DNA being present in seed washings at a relatively high level. The inability to detect *Pe* DNA in CP 8 is difficult to interpret as the DNA from all four spinach lots was of similar quality, the qPCR assay (Feng *et al.,* 2014) has been demonstrated to be extremely sensitive and host DNA in CP 8 was estimated to be at similar concentrations to those observed in the other three seed lots. Therefore, inhibition of the PCR amplification by contaminants was unlikely to be responsible (for the lack of amplification) and a low quantity of pathogen DNA below the threshold of detection could have been the cause. It will be interesting to assess whether DNA is absent in the 50, 500 and 1000 seed sub-samples from CP 8 that are scheduled for testing during subsequent stages in the project. This work will test different sample sizes, helping to inform on the optimum number of seed to screen to ensure effective detection and quantification of *Pb/Pe*. Further visual and microscopic analyses will provide greater insights into the levels of internal and external contamination of basil and spinach seed by DM, particularly any variation in the quantity of oospores produced.

All of the basil seed lots tested positive externally and internally for *Pb* RNA contamination. When testing for *Pb* RNA in the seed washings and internally, external levels were observed to be higher than inside the seed coat (approx. 100 times greater) and only a small quantity

of *Pb* was located inside the seed. This contrasted with the results from the DNA testing of identical seed lots where internal levels were greater compared to those found in the seed-washings. Detection of *Pb* RNA in washings was not anticipated, as it was perceived it would degrade quickly outside the seed coat. It remains to be established if the RNA detected on the outside on seeds came from viable conidia or mycelial fragments.

On comparison of the differences between DNA and RNA content in the respective basil seed-lots, it was observed that the amount of potentially viable *Pb* detected was on average 10 times less externally and between 500-1000 times less internally than the DNA levels indicated. This highlights how DNA-based testing potentially over-estimates the quantity (and therefore the associated risk) of downy mildew present in seed stocks. RNA levels were observed to be greatest in CP 3, with 100 times less RNA detected internally than DNA, but still approximately 5 times higher than the other most contaminated seed lots. The differences in RNA and DNA content between the lots indicated that the majority of the pathogen present in these samples may have been non-viable and that there was a five to ten times greater quantity of *Pb* located internally in CP 3 seed than present in the other untreated basil varieties. This was particularly interesting as CP 3 comprised heavily infested home-saved seed (cv Sweet Genovese, PE 024) and it was anticipated the lot should contain a high frequency of infected seeds resulting in the higher levels detected. *Pb* RNA levels were also observed to be high in CP 4 compared to the other untreated lots (CP 1A and 2A), demonstrating that RT-qPCR can accurately detect different levels of infection and supports the hypothesis that the basil variety may exhibit some partial resistance to *Pb*. However, the risk posed by specific levels of pathogen RNA still needs to be correlated with disease symptoms in a production environment. Unfortunately, this is difficult to achieve using grow-on or box tests and only low levels of transmission have been observed in previous testing (Lot F, C11412; PE 024), even when the presence of the pathogen has been confirmed with molecular diagnostics.

One caveat to using RT-qPCR to quantify basil downy mildew on and inside the seed appears to be the application of the technology to test the efficacy of steam treatments. We observed that internal RNA levels were equal to or greater in steam-treated seed than the levels in untreated seed from the same lots. This indicated that the treatment had no reductive effect, and actually led to an increase in the amount of pathogen RNA, and hence potentially over-estimated the amount of *Pb* present in lots CP 2A and 2B. One explanation for this could have been an upregulation in *Pb* gene activity in response to heat stress which could have resulted in increased levels of transcription in the genes that were targeted for qPCR.

All four spinach seed lots screened tested positive externally and internally for *Pe* RNA. Levels of *Pe* RNA were observed to be approximately 10 times less than the quantity of DNA detected in the washings in CP5, 7 and 8. The level of *Pe* RNA detected inside the seed lots was also reduced compared to the amount of DNA detected from identical lots with approximately ten times less RNA in CP 5 and up to a thousand times less in CP 6 & 7. This reiterated the limitations of DNA testing for distinguishing between both viable and non-viable downy mildew, and how this can lead to an over-estimation in quantifying the amount of a live pathogen present in seed samples. Despite repeatedly testing negative for *Pe* DNA, RNA was detected in CP 8 at higher levels than the other three lots tested indicating a greater quantity of the pathogen was present. This finding was difficult to interpret as it would be expected that DNA could also be amplified from samples testing positive for *Pe* RNA and this was not the case.

QPCR and RT-qPCR testing of contaminated seed lots has demonstrated that it is possible to detect and distinguish between differences in the level of viable downy mildew present in basil and spinach seed, respectively. Although qPCR and RT-qPCR have both been demonstrated to be sensitive and highly specific methods to detect *Pb* and *Pe* DNA, RNA appears to be a better template for accurately quantifying viable downy mildew as it is only transcribed from living cells.

Conversely, use of qPCR to detect DNA can lead to over-estimations in the amount of pathogen present by selecting for templates from both live and dead cells. Nevertheless, it will be necessary to validate the RT-qPCR approach in contaminated seed lots using parallel grow-on testing in order to establish the level of risk posed by different seed lots, although this approach can already help to improve the capability of discerning whether samples are heavily infected. RT-qPCR was unfortunately not able to demonstrate that steam was effective in reducing contamination in basil seeds due increases in the level of *Pb* RNA in the treated samples tested so far. This indicates that RT-qPCR may have limitations for testing thermally treated seed, however only two independent samples have been screened to date and a greater number of samples will need to be inspected to assess if this is a common occurrence in treated lots. Additionally, it may be feasible to select a target gene that is not upregulated in response to steam treatment as was seemingly the case for the ITS2 region selected in our experiments.

We have previously demonstrated how Propidium Monoazide (PMA) can be used in conjunction with qPCR to distinguish between differences in the amount of viable *Pb* DNA in a sample, by increasing the number of qPCR cycles in samples where the photolytic dye can penetrate dead cells (AHDB CP 184 annual report 2019). The approach will now be applied to basil and spinach seed washings from the same seed lots to investigate if it is possible to

detect differences in the amount of viable *Pb* and *Pe*, and also if it is possible to detect differences in the viability of oospores present on/in the testa (of spinach seed).

Conclusions

- Three viable isolates of *Bremia lactucae* were obtained and typed using IBEB differential varieties and all appeared to closely resemble IBEB race BI:24EU. These isolates were stored for fungicide resistance testing in 2021.
- Molecular tests have been demonstrated to be capable of detecting and quantifying small amounts basil and spinach downy mildew (DNA/RNA) both externally and inside contaminated seed lots.
- Seed lots containing low levels of downy mildew (DNA/RNA) still potentially pose a high risk for growers
- Across the seed-lots tested a greater quantity of downy mildew DNA was detected inside the seed coat compared to seed washings.
- Heat-treated basil seed lots contained approximately 50% less downy mildew DNA than untreated samples from identical lots, indicating that heat-treatment reduces the total pathogen load.
- RNA levels are generally considered to be more representative of the amount of viable pathogen present than DNA levels. In both washings and inside seed RNA levels were generally ten times less than DNA levels, showing that detection of pathogen DNA levels can lead to over-estimation of the amount of potentially viable downy mildew. Nevertheless, RNA levels increased after heat-treatment relative to untreated seed indicating a potential response in the pathogen to heat and that RNA may remain viable even when downy mildew has been neutralised. More work is needed to clarify this situation.
- Results of the work carried out on the project have been presented in 5 oral presentations at Grower meetings and AHDB webinars.

Knowledge and Technology Transfer

Wood, T (2020) Oral presentation: *Seed-borne inoculum for basil downy mildew: detection, incidence and transmission.* Basil Downy Mildew Workshop, Stockbridge Technology Centre, Cawood 5th March 2020.

Pettitt, T (2020) Oral presentation: *Risk factors for downy mildew and potential for use of decision support*. Basil Downy Mildew Workshop, Stockbridge Technology Centre, Cawood 5th March 2020

https://projectblue.blob.core.windows.net/media/Default/Horticulture/Basil%20Handout%20for%20web.pdf

Pettitt, T. (2020) Oral presentation: *Oomycete diseases control – guiding principles and practical application.* AHDB Webinar 'Prevent, detect and control oomycete crop diseases', 23rd July 2020.

https://ahdb.org.uk/events/prevent-detect-and-control-oomycete-crop-diseases

Wood, T. (2020). *Oral presentation: Downy Mildew and Blight Control Strategies – Basil seed-borne infection update*. British Herbs Trade Association, Technical Meeting 5th November 2020.

Lees, AK. (2020). Oral presentation to BLSA Protected Outdoor & Baby Leaf R&D committee meeting 18th November 2020.

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