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Project number:	CP 184
Project leader:	Tim Pettitt, Eden Project Learning (EPL) Alison Lees, (JHI) Tom Wood, (NIAB) Erika Wedgwood, (RSK ADAS Ltd)
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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

Integrated management is now essential for the effective management of downy mildews on horticultural crops. Reported here is the first year of work on a project that seeks to bring together elements of advanced diagnostic study with knowledge on current treatments and possibilities for using and adapting decision support systems/tools (DSS/DST) to painlessly maximise integrated management possibilities.

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.

Integrated pest and disease management 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 first year's progress with reviews of pathogen biology, dissemination of inoculum and the use of elicitors in disease management, progress with fungicide sensitivity monitoring as well as, assessing pathogen races and monitoring disease risks, and progress with PMA qPCR for determination of viable seed-borne infections.

Summary

Integrated management is essential for the effective management of downy mildews on horticultural crops. This amounts to careful management of all resources available to achieve a good result by accumulation of partial- or marginal-gains, including agronomy, use of resistant or tolerant varieties where available/suitable, of clean planting materials or seeds, manipulation of growing environments and irrigation as well as careful use, alternation and timing of fungicides and/or bioprotectants. This project is focussed on improving the possibilities for integrated management and developing best practice guides by: a) reviewing and collating information on potentially exploitable disease biology, on fungicides and elicitors still available (and any possibly in the future), and on disease forecasts and decision support

tools, b) developing and improving detection diagnostic procedures to screen seed for infection to help cut this significant source of disease and c) developing molecular detection and quantitation of *Bremia lactucae* as well as consolidating and building on knowledge of markers for traits like fungicide resistance in *B. lactucae* populations, and d) developing fungicide sensitivity test protocols to check pathogen populations for fungicide resistance.

In our first year of studies, the review work is largely completed and showing some useful areas where IPM may be refined and decision support tools more widely adapted. The review of elicitors has shown that there is promise of some useful activity in these materials that should be trialled for potential use as supporting components of integrated management strategies. PCR detection of *Peronospora belbahrii* (Pb basil DM) and *Peronospora effusa* (spinach DM) has been optimised and a new procedure PMA-PCR that can distinguish between living and dead pathogen has provided some promising initial results with Pb. The first sets of fungicide sensitivity assessments have revealed variable performance with Metalaxyl showing signs of widespread resistance, as might be expected, whilst Mandipropamid and Dimethomorph still show reasonable performance – the important point here is that the protocols gave useful data and it is the longer term patterns and the scope for monitoring them for changes that is key for managing future resistance. Finally, information on markers has been collated in collaboration with US research colleagues and new primers have been developed and are being tested for quantitative LAMP qPCR detection of *Bremia*.

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 crops to become unmarketable (Wedgwood, *et al.,* 2016). Although elicitors can reduce disease levels, control is not usually as good as products used directly against pathogens, and effectiveness can be more variable than plant protection products, consequently financial benefits will vary widely.

Action Points

Action points are not appropriate at this stage of the project

SCIENCE SECTION

General Introduction

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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 first year's progress with reviews of pathogen biology, dissemination of inoculum and the use of elicitors in disease management, progress with fungicide sensitivity monitoring as well as, assessing pathogen races and monitoring disease risks, and progress with PMA qPCR for determination of viable seed-borne infections.

Review work: Aspects of downy mildew biology and other information with relevance to development of integrated disease management:

Overview

A comprehensive review of the aerial oomycetes, assessing management or control options for UK production horticulture was recently completed for AHDB (Wedgwood *et al*., 2016). This report concluded that there are fundamental gaps in our understanding of this group of plant pathogens that need to be filled to help the further development of integrated disease management. A key area identified centres on the initiation of new epidemics and the role of overwintering inoculum such as oospores and systemic infections of structures such as seeds, their viability under field conditions, their capacity for germination and importance in initiating of new infections and epidemics. Also identified was a paucity of information on survival of airborne inoculum (sporangia-often referred to as 'conidia') as well as the possibility of water-borne disease spread.

The review component of the current project explores these factors plus other aspects of pathogen biology of potential importance for the development of integrated disease management. Fourteen species of importance to UK horticultural production have been selected for special although not exclusive attention (see Table 1). The downy mildews are obligate biotrophs, which means that they cannot grow or sustain themselves without a living host, and this makes working with them to ascertain optima for propagule germination and sporulation as well as sourcing and maintaining infective isolates less straightforward than

Table 1. Downy mildew species considered in this review: their hosts and selected aspects of their biology potentially important for developing IPM for their management

Pathogen	Crop	Oospores	Seedborne infection	Debris/ Soilborne infection	Sporangia (conidia)	Zoospores	Survival of sporangia/ conidia	Latent period (days)	Systemic infection	Infection		
										LWD	Temp (°C)	RH%
Bremia lactucae	Lettuce	+	(+)	+	+	-	>12h	7-9	-	>2h	5-23	>85
Hyaloperonspora parasitica	Matthiola/ Stocks	+	+?	+	+	-	>3days	5-6	+?	>4h	10-21	>90
Peronospora aquilegiicola	Aquilegia	+	+?	+?	+	-	?	>3	?	10h optimum	5-20	>70
Peronospora belbahri	Basil	+	+[b]	+?	+	-	<3 days	5-10	+	≥4h	5-28	>85
Peronospora destructor	Onions	+	+	+	+	-	1-3days	9-16	+	2-4h	8-12	>93
Peronospora effusa	Spinach	+	+	+	+	-	Several days	7-10	+	>3h	15-21	>90
Peronospora grisea	Hebe	+	-[a]	+	+	-	?	?	?	>6h[c]	10-22[c]	100[c]
Peronospora potentillae* var. *gei	Geum	?	?	+?	+	-	?	?	?	? 'wet'	? 'cool'	? 'High'
Peronospora sparsa	Rose	+	-[a]	+	+[sparse!]	-	?	4-7	+	>2h	15-20	>90
Peronospora viciae* var. *pisi	Peas	+	+[†]	+	+	-	3-5 days	6-10	?	>4h (6h)	12-20	>91
Peronospora violae	Viola	+	?	+?	+	-	?	?	?	? 'wet'	? 'cool'	? 'High'
Plasmopara obducens	Impatiens	+	+?	+	+	+	?	?	?	? 'wet'	15-23	? 'High'
Plasmopara petroselini	Parsley	+	+	+	+	+	?	?	?	>1h but strongly seasonal	? 'Low temps'	? 'High'
Phytophthora infestans	Tomatoes	(+)	-[a]	+	+	+	2-8 d wet ≤24h dry	3-6	+	>2h	13-23	100

[a] – Possibly infections *via* contaminated cutting tissues e.g. in UK 2018 *P. infestans* outbreak in tomatoes (Pettitt *et al*., 2018). [b] – Contested for arid production environments (Falach-Block *et al*., 2019). [c] – HRI Efford dehumidification trials – unpublished data. † - likely to result in seed mortality. LWD – leaf wetness duration.

References: Aegerter *et al*. (2003); Bashi & Aylor (1983); Choudhury *et al*. (2016; 2018); Cohen *et al*. (2017); Falach-Block *et al*. (2019); Foss et al. (2005), Garibaldi *et al*. (2004); Gilles *et al*. (2004); Gómez (2014); Gómez & Filgueira (2012); Hildebrand & Sutton (1982; 1984b); Inaba *et al*. (1983); Jafar (1963); Jennings (2011); Jennings *et al*. (2016; 2017);Kandel *et al*. (2019); Kennedy & Wakeham (2008); Koike *et al*. (2006); Kröber (1970); Lee & Rose (2010); Lyon *et al*. (2016); Mariette *et al*. (2016); Maude (2006); Minchinton (1998); Minchinton *et al*. (2006); Neog *et al*. (2015); Palti (1989); Pegg & Mence (1970); Porter & Johnson (2004); Scherm & van Bruggen (1994a); Shishkoff (2019); Soylu *et al*. (2009); Srivastava & Singh (1988); Subbarao *et al*. (2018); Sunseri *et al*. (2002); Thines *et al*. (2019); van den Bosch *et al*. (1988); Van der Gaag & Frinking (1997c); Wallace *et al*. (2018); Wedgwood *et al*. (2016), Whipps & Linfield (1987), Wright *et al*. (2014); Wu *et al*. (2000); Wyenandt *et al*. (2015), Xu & Pettitt (2004, 2017); Zhang *et al*. (2019).

other groups of phytopathogens. Biotrophy is a life strategy that requires specialised adaptation of the pathogen to its host and as a consequence there are many species of downy mildew strongly coevolved with their specific host genera/families. Despite this variation, the downy mildews considered respond in broadly very similar ways to environmental parameters (Table 1) and there are relatively large amounts of observational data published on some of the species considered here (*B. lactucae, P. belbharii* and *P. destructor*).

Bremia lactucae **on lettuce:**

Downy mildew in lettuce caused by *B. lactucae* is one of the more heavily researched aerial oomycete pathogens. This disease has a worldwide distribution and causes extensive losses internationally (Subbarao *et al*., 2017). Under UK conditions it can produce abundant resilient overwintering oospores in infected tissues (Michelmore & Ingram, 1980), which can survive in soil and debris between crops and initiate epidemics in new plantings (Gustafsson *et al*., 1985, van Hese *et al*., 2016) although the importance of this relative to spread by sporangia is not always clear. Disease rapidly builds up and is widely disseminated by asexual sporangia generated in repeated cycles under favourable conditions and spread by wind and rain-splash (Fletcher, 1976). Seedborne infection may be possible but is not generally considered important in lettuce downy mildew. Disease management has been achieved by use of resistant varieties in combination with fungicide sprays in regimes refined in the 1980s in the face of new virulence genes and the development of resistance to phenylamides (*sub group* acylalanines e.g. Metalaxyl see Table 2) in *Bremia* populations (Crute, 1984, 1987). Often spray intervals are at set calendar dates but increasingly timings are being decided by meteorological parameters based on the influence of these on pathogen spore germination and infection. Key parameters that have been investigated in this regard are temperature, relative humidity (RH%), rainfall, leaf wetness duration (LWD) and aspects of light. The LWD has a very strong impact whilst the temperature optimum for germination and subsequent infection is broad (5-20^{o}C at a LWD of ≥4h), with the most germination after ≥12h LWD at 5^{o}C, and after ≥4h at 10-15^{o}C, poorest at 25^{o}C and no germination or infection at 30^{o}C (Scherm & van Bruggen, 1993). Breaking the LWD in UK protected lettuce crops using increased night heating was shown to reduce downy mildew almost as effectively as metalaxyl fungicide treatments (in the absence of fungicide resistance), although the expense of heating was considered uneconomic at that time (Morgan, 1984) – a consideration that might change if a situation develops where key fungicides for control are no longer available? Nevertheless, this approach might still be worth revisiting for integrated management strategies in the light of successful results achieved with integrated heat-lift venting treatments for *Botrytis* management in protected bedding plants (Pettitt, 2002/3,

unpublished). In this work, two factors were important, a) the capacity for spores/spore germlings to recover (or otherwise) after drying and b) their initial level of hydration. This information appears to be currently unavailable for *B. lactucae*, although indications are that sporangia can survive adverse (drying) humidity and that survival on leaf surfaces is more influenced by temperature than RH% with spores surviving for 12h at 23°C but only for 2-5h at 31°C at 33 and 76% RH (Wu *et al*., 2000). The apparently small influence of RH% may be as a consequence of transpiration causing the air at the leaf surface to be close to saturation Harrison (1992). The direct impact of spore hydration on survival and germination has not been measured although in DEFRA-funded research at Warwick HRI (DEFRA, 2005, HH3217TFV), infection of lettuce hypocotyls was rapid (<0.5h on average compared to the more widely observed 2h minimum - Scherm & van Bruggen, 1993) following inoculations with aqueous suspensions of fully hydrated sporangia. No studies have been made of the impact of drying on the viability/infectivity of partially germinated sporangia of *B. lactucae*.

Analysis of disease in relation to weather variables in California identified morning LWD as the most important indicative variable, with a 4h median morning (between 0600 and 1000 hrs Pacific Standard Time) LWD identifying infection days (Scherm & van Bruggen, 1994b). A mechanistic model based on these observations, using numerical weather forecasts to predict infection and non-infection days, was developed and tested as a decision support system (DSS) providing spray advisories and gave a significant reduction in the number of sprays applied in Californian field conditions with no drop in disease control (Scherm *et al*., 1995). The biological validity of this model was supported by observations that infection could immediately follow sporulation on mornings with extended LWD and that spore release is initiated in the morning period (Scherm & van Bruggen, 1995). Also, whilst the effects of light are considered in detail elsewhere in this AHDB project, it is important to mention here that daytime light (especially the UV component) was found to be inhibitory to sporulation and spore viability (Wu *et al*., 2000), demonstrating again the validity of considering periods of LWD of highest importance for mildew sporulation to be those that occur in the dark or more specifically in the period before daybreak. Further improvements were made to the Californian model by Wu *et al*. (2002); 1) shortening the morning LWD period to 3h, 2) adjusting the commencement of its timing from 0600 hrs to the sunrise as determined by photon flux density rising above 41 µmol m^{-2} S^{-1} or the solar radiation above 8 W m^{-2} (depending on which measure was available), and 3) accounting for negative temperature effects on infection by assuming no infection when temperature was greater than 20°C during the 3h LWD or was greater than 22°C during a subsequent (wet or dry) 4h period.

Kushalappa (2001) argued that whilst morning LWD provides an excellent predictor for the Californian situation, this model might have limited applicability elsewhere (e.g. Canada)

because by focusing on spore release and infection criteria (the primary processes impacted by the morning LWD period), it does not consider *all* of the components of the *B. lactucae* disease process – most importantly sporulation (although this was assessed by Wu *et al*., 2001). To address this point, the BREMCAST model considered weather variables in relation to sporulation, spore release and infection (Kushalappa, 2001). Advisories provided by this forecasting model were tested in Canadian field crops by Hovius *et al*. (2007) using sprays of protectant fungicide Mancozeb compared with scheduled weekly applications of the same treatment. Advisories were triggered by the recording of a 'sporulation-infection period' (SIP) of a LWD from 0300 -1000 hrs with a temperature between 5 and 20°C, and sprays were applied just prior to expected symptom development based on the estimated latency/incubation period (i.e. the time between infection and first symptom appearance), but not within 7 days of a previous spray treatment. The latency period was estimated by the procedure of (Scherm & van Bruggen, 1994a) at 115 degree days (with a base temperature of 0°C, calculated as the daily maximum temperature plus the daily minimum temperature divided by two, and accumulated from 0600 hrs on the day of the SIP), or 8 days – whichever was the longest. These trials worked and the number of sprays was generally reduced by one or two per crop, except when disease pressure was consistently high, with no difference in efficacy compared to the routine weekly spray schedule. A perhaps riskier schedule was also tested which relied upon the curative properties of a metalaxyl-M plus mancozeb fungicide combination which was applied at the time of anticipated sporulation, 135 degree days after a SIP was recorded, as a post-latent period treatment. Although reducing the number of sprays and comparing well with a metalaxyl seedling drench treatment (Hovius et al., 2007), this seems an ill-advised approach from the perspective of encouraging fungicide resistance unless more fungicides with curative action against *Bremia* become available to allow alternation of actives within the spray programme.

Both the DSS models described operate on the assumption that most spores are released in the morning hours and that a large proportion of these immediately go on to germinate and infect. However, measurements of airborne sporangia in Ontario (Canada) crops by Carisse & Philion (2002) using 7-day volumetric spore traps revealed three different patterns of spore release: the most frequent pattern confirmed the assumption of the models above with a peak of spore numbers (representing about 75% of the daily catch) at about 10:00 hrs, less frequent was a similar pattern but with a later peak, whilst the third was a completely random pattern and was associated with days when low numbers of spores were airborne. Further measurements of airborne sporangia concentrations in relation to infection, both in controlled environments and in field crops in Quebec (Canada), demonstrated that whilst the general pattern of spore release was similar to that observed in California, only approximately 50% of

the total daily inoculum was airborne by 10:00hrs and that a further 25% or so was released by 14:00hrs (Fall *et al*., 2015). This study also revealed that under Canadian conditions, a 2h LWD was sufficient to initiate infections and that spore survival rates meant that more accurate spray advisories were provided by considering LWDs both in the morning and evening (18:00 – 22:00hrs) periods. In addition, Fall *et al*. (2015) calculated the infection efficiency of airborne inoculum and found that a daily airborne inoculum concentration required to cause 1 lesion leaf^{-1} was 1 sporangium (conidium) m^{-3} in their controlled environment experiments and 10-14 sporangia m^{-3} in the field (under appropriately conducive environmental conditions of temperature and moisture). This disease threshold information needs to be verified for other lettuce growing areas and combined with improved weather metrics provides the basis for more reliable DSS.

Detection of pathogen inoculum is obviously of key importance in honing the reliability of DSS and this is provided to a limited extent in the BREMCAST system where information on presence of lesions in crops made available from routine crop scouting provides a route to delivering a spray advisory (Kushalappa, 2001). Fall *et al*. (2016) recently developed a quantitative dynamic model to simulate airborne *B. lactucae* sporangium concentrations (simulating trend and temporal change in numbers as opposed to straight predictions of presence/absence) based on weather parameters. This model provided a reasonably high degree of accuracy. Nevertheless, there were shortcomings with simulations not matching trapped spore counts during one test season. Possibly this situation arises from problems with quantifying the number of sporulating lesions under a given set of weather conditions and the sensitivity of the simulation is strongly reliant on the good quality of the meteorological data used. Use of a simulation of infective inoculum concentration for DSS does carry the potential advantages over direct trapping and assessment of spores present of being immediate and not being susceptible to the variations due to the positioning of traps. Probably an ideal scenario will be the use of both a simulation and actual spore trap data combined to reduce the need for multiple spore traps to give reliable estimates of spore populations. As yet, current DSS need to be adjusted if they are to take airborne inoculum concentration into consideration.

Precise direct quantification of airborne inoculum can be achieved using conventional Burkard 7-day spore traps and counts on microscope slides although the equipment is expensive, there is a delay (the '7-day spore trap' name is a clue), whilst the counts are laborious and take specialist skill to discern and properly count spore types. However, alternative spore trap types and methods of diagnosing and quantifying inoculum are available. Two main routes to diagnosis and quantification are possible; immunodiagnostic procedures or qPCR. Immunodiagnostic procedures have been explored and developed more fully for onion and

brassica downy mildew and will be covered in more depth in the sections of this report covering those pathogens. A monoclonal antibody has been raised at Fera as part of a DEFRA-funded project based at Warwick-HRI at Wellesbourne, EMA 245 raised from cell line 2H4\5H6G10, which in reactivity tests showed a high level of specificity and sensitivity to *B. lactucae* (DEFRA, 2005, HH3217TFV). If the cell line is still viable it should be possible to deploy this antibody in similar procedures to those outlined for onions and brassicas below (Wakeham, 2013). The alternative would be qPCR and a *B. lactucae* specific primer set and qPCR assay has recently been developed and optimised in the USA for use with samples collected by rotating arm impaction spore traps (Kunjeti *et al*., 2016). This procedure has been undergoing field trials in California over the last couple of seasons with a view to using it to provide a new generation of DSS spray advisories and early assessments have shown it capable of reducing the number of sprays by an average of 1.7 over regular once per week schedules (Klosterman *et al*. (2019). Whilst this approach is showing great promise, it still has the potential shortcomings of being a relatively costly procedure, requiring expensive reagents and equipment and skilled operators plus the possibility, with such a sensitive assay, of cross-contamination of samples (seen in commercialisation of qPCR in other systems e.g. powdery mildew in grapes: Reiger 2013; Wakeham & Pettitt, 2017). An additional concern is that the qPCR process as described by Kunjeti et al. (2016) would be unable to discern between viable and non-viable spores and yet the loss of spore viability in daylight has been shown to be in part responsible for the patterns of infection seen in California (Wu et al., 2000). This concern may be possibly addressed if the use of PMA pre-treatments for qPCR described in the seedborne infection section of this report can successfully discern between living and dead downy mildew biomass.

***Peronospora belbahrii* in Basil:**

This pathogen in its current aggressive form, firmly identified as a new species *Peronospora belbahrii*, first appeared and rapidly became widely distributed in basil (*Ocimum basilicum*) production around the world in the first decade of this century (see review of Cohen *et al.* (2017) for a comprehensive list of international first outbreak reports). Since its recent appearance in basil crops and, as a consequence of its rapid worldwide spread, seedborne infection by *Peronospora belbahrii* has been considered highly likely (Garibaldi *et al*., 2004; Farahani-Kofoet *et al*., 2012; Wyenandt *et al*., 2018). Seeds have been tested for presence of *P. belbahrii* by PCR, and sporangiophores and sporangia were seen in seed wash-water from PCR-positive seed samples, although no oospores or deep-seated infections (e.g. in seed embryos) have yet been recorded either from washings or microscopic examination of the same seed samples (Wyenandt *et al*., 2018; Jennings *et al*., 2017). Nevertheless, Israeli

research (Falach Block *et al*., 2019) asserts that despite there often being many sporangia imbedded in the mucin layer secreted on basil seed-coats and readily detectable by PCR, these spores lose their viability under hot, dry Israeli conditions. After conducting extensive trials over several years of PCR positive seed-lots under conditions favourable to downy mildew development, these authors observed no disease and concluded that seedborne transmission of *P. belbahrii* does not happen under Israeli conditions. However, this does not seem to be the case under the cooler/wetter conditions in Europe where very low incidences of seed-borne disease transmission have been observed. Garibaldi *et al*. (2004) saw incidences of infected plants of 0.33-0.66% in 4 out of 16 seed-lots they assessed, whilst more recently Jennings *et al*. (2019) assessed two UK seed samples and recorded 0.4% and 1.3% infection.

Disease when it appears can rapidly spread from the small numbers of initial infections possible from low incidences of seedborne transmission and appear to 'suddenly' become widespread. This may in part be due to the cryptic nature of early symptoms but may also be the result of systemic infections (Farahani-Kofoet *et al*., 2012; Falach-Block *et al*., 2019) leading to multiple sporulating lesions emanating from single infection points. To maintain control of an epidemic it is important to carefully monitor crops and to immediately remove plants showing initial symptoms which consist of slight chlorotic patches appearing on the upper leaf surfaces and which can be mistaken for Mg deficiency (Budge, 2011). Based on their infection studies Zhang *et al* (2019) suggested that scouting fields for initial infections is likely to be an unreliable method of initiating epidemic management and they recommended development of PCR-based detection based around some form of air sampling. This could possibly be based around similar procedures to those being developed and used against *Peronospora effusa* in spinach and lettuce (Klosterman *et al*., 2014, 2019; Kunjeti *et al*., 2016 - see below), although given an appropriate antibody probe, immunodiagnostic systems (Wakeham, 2013) would likely be equally effective and maybe better suited to in-field assessments.

Like other downy mildews, the severity of infection by *P. belbahrii* is dependent upon LWD, temperature and inoculum concentration (Cohen *et al*., 2017). High RH% (>85%, optimal 98-100%) is needed for sporulation and infection (Wyenandt *et al*., 2015) and sporangial germination and infection occur between 5 and 28°C (Cohen *et al*., 2017), with an optimum of 20°C (Garibaldi *et al*., 2007; Wyenandt *et al*, 2015; Jennings *et al*., 2017). Sporulation has a slightly narrower temperature range (10-26°C, optimal at 18°C) and occurs only in the dark (Cohen *et al*., 2013a & 2017). This narrower band of temperatures may explain the observation of Garibaldi *et al*. (2007) of no disease at <12°C or >27°C. Using data generated from inoculations of plants maintained under constant conditions in growth cabinets, Jennings

et al. (2017) have generated a very useful guide to the disease risks associated with different combinations of temperature and RH% (Jennings *et al*., 2017: Figure 2, p 25), which might form the basis of a simple DSS.

Cohen & Ben-Naim (2016) observed that if healthy potted plants were placed for 1 night in a net house containing an infected crop, then transferred and incubated in isolation, they became diseased. This observation indicates that *P. belbahrii* infection is capable of proceeding in same way as Bremia on lettuce whereby sporulation and infection can occur as a continuous process all in one go, given the right conditions of dark, high RH% period for sporulation followed by morning spore release and a sufficient LWD to initiate germination and penetration of the host plant's tissues (see above). Bearing this in mind it may be worth considering adapting a forecast system from another mildew disease system such as BREMCAST or DOWNCAST (see section on DSS below). The study of Cohen & Ben-Naim (2016) describes the successful development of nocturnal fanning treatments to supress downy mildew sporulation. This type of treatment relies on the ability of the air movement from the fan to reduce the moisture level at the leaf surface and when the air is generally very moist it may cease to be effective and even assist the movement of the target pathogen within the crop as was seen in some UK crops of *Eustoma* infected with the downy mildew *Peronospora chlorae*, although this situation may also have been the result of fans sometimes running during daylight hours when spore release was more likely to occur (Pettitt, 2001 unpublished). An interesting result of Cohen & Ben-Naim's (2016) study was the impact on infection of the timing of a 10-minute drying period within a 9 h LWD. If imposed after 1h, the drying period had no effect and after 2h it gave a slight reduction in infection but after 3h and after 4h it reduced infection from 70% to 10% and 12% respectively, then after 5, 6, 7 and 8h the drying period again had no impact. This result implies that a drying period applied after about 3-4h LWD curtailed germination/infection and may even have been lethal to infection structures and indicates that the use of a heat lift/venting treatment linked to environmental parameters such as leaf vs air dew point or LWD, as successfully developed with Botrytis in pack-bedding and potted ornamentals (O'Neill *et al*, 2002), might be a useful component in an integrated control program for basil downy mildew.

Peronospora destructor in onions

P. destructor has very similar surface wetness (LWD) and relative humidity requirements for sporulation and infection to *Bremia* and *P. belbahrii* (Table 1). Sporangia are also similarly mostly released under the influence of solar radiation and reducing humidity at daybreak (Leach *et al*., 1982). In common with *P. belbahrii*, onion downy mildew infection has been

shown to be disrupted by alternating periods of wet and dry conditions during the night and this is related to the increased mortality of *P.destructor* spores under these conditions (Hildebrand & Sutton, 1984a). Interestingly, the effect cycles of wet and dry did not appear to affect spores' viability/survival during the day, nevertheless the phenomenon requires further investigation as it may be of considerable use for disease management by timed regulation of LWD, especially in protected crops. *P. destructor* is one of a small number of downy mildew pathogens to infect a monocotyledonous host and onion downy mildew is characterised by generally longer latent periods than the similar intensively studied diseases (Table 1; Hildebrand & Sutton, 1982). In addition to longer latent periods *P. destructor* infection and sporulation are optimal at slightly lower temperatures to *Bremia* and *P. belbahrii* (Hildebrand & Sutton, 1984b). Environmental conditions conducive to onion mildew epidemics were studied in detail by Hildebrand & Sutton in the 1980s (Hildebrand & Sutton, 1982; 1984a; 1984b; 1984c) and this work underpinned later work developing effective decision support models for this disease.

The possibility of reducing the number of routine sprays or improving their timing by taking a bioclimatological approach to forecasting downy mildew disease in onion crops as part of an integrated control strategy was first recognised by de Weille (1975), and the first such model, DOWNCAST' was developed in Canada Jesperton & Sutton (1987). The model predicted a sporulation-infection period when environmental conditions were conducive to sporulation, dispersal, survival and infection and was found to be effective in predicting infection in Australian crops of onions and, interestingly, lettuce reducing the numbers of sprays needed per crop by 1 to 2 (Fitzgerald & O'Brien, 1994; Minchinton *et al*. 2010). Under the wetter and cooler North-Western European climates sporulation predictions by DOWNCAST and DOWNCAST modifications (e.g. ONIMIL Battilani *et al*., 1996) were found to be inaccurate (de Visser, 1998; Gilles *et al*., 2004). In response to this shortfall improved models were developed in Germany (ZWIPERO – German Weather Service model, Friedrich *et al*., 2003) and the UK (MILIONCAST, Gilles *et al*., 2004). These models improved the accuracy of sporulation, spore release, survival and infection predictions, although plant development, disease susceptibility and actual inoculum present in the field were not accounted for. The effectiveness of MILIONCAST has been further improved by augmenting disease risk predictions by use of low-cost in-field spore detection/quantitation assays (Kennedy & Wakeham, 2008; Wakeham *et al*., 2012), although further refinements are needed to allow for changes in infection efficiency (Wakeham *et al*., 2016), meanwhile the MILIONCAST model has been redeveloped for improved accessibility in AHDB-funded work (CP 186, Van Den Berg, 2020) and is available via the Crop Monitor website (http://www.cropmonitor.co.uk).

Other representative downy mildew pathogens from UK horticultural crops

Of the remaining species of downy mildew selected for study in this project (Table 1), none has been studied in the same depth as *Bremia lactucae* in lettuce and *Peronospora destructor* in onions. Detailed information is only available for mildew in spinach, rose, tomato (*Phytophthora infestans*) and to a lesser extent, peas, stocks, aquilegia, impatiens and hebe, whilst very little specific biological information of use for IPM/DSS is available for downy mildews of geum, viola and parsley (Table 1). *Peronospora effusa* in spinach has recently been reviewed (Kandel *et al*., 2019) and whilst there is much generic information presented, this review is still rather thin on actual *P. effusa* data. For example, the presence of oospores on spinach seeds was demonstrated some time ago by Inaba *et al.* (1983), but has only very recently started to receive serious scrutiny as a potential source of primary infections and disease spread with firm conclusions still to be drawn (Kandel *et al*., 2019). Little is published about sporulation of *P. effusa*, although the optimum sporulation conditions for the closely related species *P. farinosa* (only recently separated, Choi *et al*., 2007) recorded on sugar beet as 12ºC and RH of ≥85% (Byford, 1981). More is known about sporangial germination and infection where optima have been defined and latent periods calculated (Choudhury *et al*., 2018; van den Bosch *et al*., 1988). Choudhury *et al*. (2018) found that in line with other mildews blue light inhibited sporangial germination, but also made the additional observation that germination over a wide range of temperatures, declined in continuous darkness on water agar (conditions that might otherwise be considered stimulatory). Further work has been done on spore release, dispersal and detection (Choudhury *et al*., 2016; 2017; Klosterman *et al*., 2014; 2019) and this is considered in more dept in the section of this report covering Decision Support Tools/Systems.

Rose downy mildew (*Peronospora sparsa*) was reviewed in depth by Wedgwood *et al.* (2016) and more recently by Salgado-Salazar *et al*. (2018). The optimal temperature for sporulation and infection for *P. sparsa* falls between 15 and 20ºC (Table 1: Aegerter *et al*., 2003; Filgueira & Zambrano, 2014; Kim *et al*., (2014); Mudiyanselage (2015)), although the temperature range within which some infection will occur is very broad (4-33ºC Filgueira & Zambrano (2014); 4-27ºC Minchinton, 1998). Sporulation is naturally rather sparse (hence the name), and often difficult to see, but under optimal conditions it has a high infection efficiency and disease is optimal with high humidity and LWD >3h with high average rainfall Kim *et al*. 2014). There has been sufficient data generated for disease development that several reasonable decision support models have been developed (see DSS/DST section below).

Not much of substance has been published on the biology of tomato late blight caused by *Phytopthora infestans* since the review of Wedgwood et al. (2016). In 2018, an outbreak of the disease occurred in UK protected crops that could be traced to the original propagules – one of the sources of primary infection outlined by Wedgwood *et al.* (2016). However, during this disease outbreak symptoms were unusual, consisting of a steadily progressing light tan-coloured stem necrosis that progressed up the main stems from at or near the graft union but did not cause vascular collapse and shoot death and showed no signs of secondary spread (Pettitt *et al.*, 2019). Samples of affected stem were incubated at 20°C over a range of RH% and sporulation rapidly occurred at RH ≥95%. In addition, infections by the same genotype (EU_39_A1) in outdoor tomato crops in other parts of the EU showed more typical late blight symptoms including widespread sporulation, aggressive necrotic lesions, infected leaves and fruit, and shoot death. These observations suggest that the disease may have successfully been kept in check by the relatively dry climate inside most UK glass during the 2018 season. Use was also made of the Blight Cast DST which provided warnings of potential Hutton Periods during which greenhouse staff were extra vigilant and in the case of one older greenhouse a very small amount of sporulation was found and effectively treated with an application of Ranman Top (cyazofamid/L).

Soil and debris-borne oospores are generally considered the main source of primary inoculum for outbreaks of downy mildew in peas caused by *Peronospora viciae* var. *pisi* (van der Gaag & Frinking, 1997a; 1997b; 1997c; Chang *et al*. 2013; Stoddard *et al*., 2010). The best set of data on the response of this pathogen to temperature, moisture and light conditions is still the study by Pegg & Mence (1970), which Wedgwood *et al.* (2016) rightly points out, needs to be re-evaluated in the light of subsequent determination of distinct races within *P. viciae* var. *pisi.* Wedgwood *et al*. (2016) also point out that currently there are no disease risk forecasting models developed for pea downy mildew, although there might be the possibility of at least drawing up a simple risk table similar to that devised by Jennings & Thorp (2016) for downy mildew on aquilegia (*Peronospora aquilegiicola*). A relatively recent disease problem (Denton *et al*., 2015;Thines *et al*., 2019), *P. aquilegiicola* is likely to be seedborne although this has not been conclusively proved (Jennings *et al*., 2016; RHS 2019 website) and is favoured by extended periods of LWD, high humidities (RH% 90-100%) and temperatures in the range 5-20°C (optimal at 15-20°C, see Table 1).

Surprisingly little of the epidemiology of the downy mildew of Hebe has been quantified, bar some simple estimates of temperature optima for infection and sporulation (see Table 1). Commonly referred to as *Peronospora grisea*, this name is currently under review and only correctly refers to the downy mildew pathogen on related host species *Veronica beccabunga* (Thines & Choi, 2015). Apart from an excellent grower leaflet (Mason & Jennings, 2019) and

the work of Neog *et al*. (2015) modelling epidemics in rapeseed little valuable epidemiological information is published on *H. parasitica* and virtually no non-anecdotal evidence appears to exist for *P. potentillae* var*. gei* and *P. violae* on Geum and Viola respectively.

The mildews of Impatiens and Parsley are caused by species of *Plasmopara* (*P. obducens* and *P. petroselini* respectively), a genus that produces motile infective zoospores. *Plasmopara* sporangia usually release zoospores when they land on a wet plant surface, and these motile propagules have the potential to generate multiple infection sites from a single sporangium. A zoosporic phase in the infection cycle also can increase dependency upon LWD for successful infection, although it is possible that these motile spores are able to exploit free water, germinate and establish infections more rapidly than directly-germinating non-zoosporic sporangia. It's known that oospores of *Plasmopara viticola* causing downy mildew of the vines, can germinate in wet soil conditions to form a macrosporangium that releases zoospores and can thus rapidly re-establish downy mildew disease even after a period of drought (Rossi & Caffi, 2007; Rossi *et al*., 2008). Shishkoff (2019) germinated *P. obducens* oospores to produce single macrosporangia which released zoospores and were able to infect impatiens leaves over a range of temperatures from 0-10^{o}C. In the case of *P. petroselini* 1h of LWD is sufficient for successful infections to establish, although the infection process appears to be strongly temperature dependent and Wright *et al.* (2014) found that despite prolonged periods of leaf wetness and high humidity in summer months, infection of parsley by *P. petroselini* did not occur until autumn and a drop in average temperature.

Current situation with anti-downy mildew fungicides

For downy mildew control in the crops considered here, a total 15 fungicide active ingredients are available, representing 13 different FRAC cross-resistance groups (FRAC 2020, Table 2). The majority of these chemicals are protectants, with only three (metalaxyl-M, azoxystrobin & benthiavalicarb) showing any curative properties and all these three carry a risk of fungicide resistance that somewhat compromises their use solely as curative treatments in an integrated control program. Whilst the total of FRAC groups available is 13, individual crops each have fewer groups at their disposal (Tables 3a & 3b), with protected and outdoor peas in the worst position with two currently available, azoxystrobin and Chlorothalonil (the latter is a multi-site inhibitor used here in a mixed formulation with azoxystrobin to reduce the risks from fungicide resistance), with approval for the latter to be revoked in May this year (2020). The largest numbers of FRAC groups are available to HNS, protected and outdoor roses, as well as protected and outdoor parsley and basil. The herbs each have the same 9 groups available (Table 3b -only parsley labelled in table), of which

three carry a medium to high risk of fungicide resistance and a further three for which the resistance in downy mildew populations is unknown. The HNS sector have a slightly different range of 9 groups available (Table 3b), of which four carry a resistance risk and two are of unknown resistance reaction. Similar selections of between 7 and 8 FRAC groups are available for outdoor lettuce, leafy brassicas and spinach crops, with slightly fewer (between 4 and 7) for protected crops (Table 3a),

Table 2. List of fungicide active ingredients currently available (June 2020) for use on one or more of the downy mildew diseases listed in Table 1 above.

(*N.B*. No one crop has access to all of the fungicides listed here, and some are currently highly restricted e.g. Pea/*P. viciae* var *pisi* – restricted to Azoxystrobin and Chlorothalonil {Table 3b} and registration for the latter is due to be revoked in May 2020)

Common name (active ingredients)	FRAC No.[a]	Chemical group		Action[b]
Metalaxyl-M	4 (A1)	Phenylamides sub group: Acylalanines		Systemic with curative and protective action. Disrupts pathogen nucleic acid synthesis
Azoxystrobin	11 (C3)	Strobilurins		Systemic translaminar and protectant action having additional curative and eradicant properties. Respiration inhibitor
Propamocarb hydrochloride	28 (F4)	Carbamates		Systemic, with protective action absorbed and translocated. Lipid synthesis inhibitor.
Fosetyl-aluminium	33 (P7)	Ethyl phosphonates		Plant host defence system inducer
Benthiavalicarb	40 (H5)	Valinamide carbamates	Carbolic acid amides (CAA)	Protective and curative action with residual effects, inhibits phospholipid biosynthesis
Dimethomorph	40 (H5)	Cinnamic acid amides		Systemic with protective activity. Cellulose synthesis inhibitor.
Mandipropamid	40 (H5)	Mandelic acid amides		Systemic with protective activity. Cellulose synthesis inhibitor.
Fluopicolide	43 (B5)	Benzamides		Protectant, novel mode of action as fluopicolide delocalises spectrin-like proteins
Bacillus amyloliquefaciens D747	44 (F6)	Live bacterial preparation (*Bacillus* sp.)		Microbial disrupters of pathogen cell membranes - protectant mode of activity
Ametoctradin	45 (C8)	Triazolo-pyrimidylamines		Selective, preventative, mitochondrial respiration inhibitor
Mancozeb	M3	Dithiocarbamates		Broad spectrum, non-systemic, contact with protective action, acts by distrupting lipid metabolism. Multi-site activity.
Captan	M4	Phthalimides		Non-systemic with protective and curative action. Multi-site activity.
Chlorothalonil	M5	Chloronitriles		Non-systemic, broad-spectrum, foliar action with some protectant properties. Acts by preventing spore germination and zoospore motility. Multi-site activity.
COS-OGA	N/A	Complex comprising oligo-pectates and chito-oligosaccharides		Plant host defence system inducer
Potassium hydrogen Carbonate	N/A	Bicarbonates (Old name=potassium bicarbonate)		Protectant and eradicant, bicarbonate causes the collapse of hyphal walls and shrinkage of pathogen spores

[a]Fungicide resistance risk = High | = Medium to high | = Low to medium **All need risk management**

[b]Fungicides with curative action

Information from PPDB and FRAC (2020)

Table 3a. Fungicides currently available for downy mildew management in outdoor and protected Lettuce, Leafy Brassicas, and Spinach crops.

Product Name	MAPP No.	Formulation name	Formulation type	Outdoor Lettuce	Protected Lettuce	Outdoor L Brassicas	Protected L Brassicas	Outdoor Spinach	Protected Spinach
Amistar	18039	Azoxystrobin	Suspension concentrate	✓	✓				
Azofin Plus	18552	Azoxystrobin	Suspension concentrate	✓	✓				
Amylo X WG	17978	*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747	Water-dispersible granules			✓	✓	✓	✓
Avatar	16608	Fosetyl-aluminium/propamocarb hydrochloride	Soluble concentrate						
Captan 80 WDG	16293	Captan	Water-dispersible granules						
Clayton Belfry	18154	Azoxystrobin	Suspension concentrate	✓	✓				
Conclude AZT 250SC	18440	Azoxystrobin	Suspension concentrate	✓	✓				
Fubol Gold WG	14605	Mancozeb/metalaxyl-M	Water dispersible granules		✓		✓		
Fytosave	18433	COS-OGA	Soluble concentrate	✓	✓	✓	✓		
Infinito	16335	Fluopicolide/propamocarb hydrochloride	Suspension concentrate	✓		✓		✓	
Invader	15223	Dimethomorph/mancozeb	Water-dispersible granules	✓		✓			
Karamate Dry Flo Neotec	14632	Mancozeb	Water-dispersible granules	✓		✓			
Karma	16363	Potassium hydrogen carbonate	water soluble powder	✓	✓	✓	✓	✓	✓
Olympus	18158	Azoxystrobin/chlorothalonil	Suspension concentrate						
Pan Cradle	15923	Fosetyl-aluminium/propamocarb hydrochloride	Soluble concentrate	✓	✓				
Paraat	15445	Dimethomorph	Wettable powder	✓	✓	✓	✓	✓	
Percos	15248	Ametoctradin/dimethomorph	Suspension concentrate						
Plant Trust	15779	Fosetyl-aluminium	Encapsulated granule						
Previcur Energy	15367	Fosetyl-aluminium/propamocarb hydrochloride	Soluble concentrate	✓	✓			✓	
Promess	16008	Propamocarb hydrochloride	Soluble concentrate						
Proplant	15422	Propamocarb hydrochloride	Soluble concentrate						
Revus	17443	Mandipropamid	Suspension concentrate	✓	✓	✓	✓	✓	✓
SL 567A	12380	Metalaxyl-M	Soluble concentrate			✓	✓	✓	✓
Toran	18239	Azoxystrobin	Suspension concentrate	✓	✓				
Valbon	14868	Benthiavalicarb-isopropyl/mancozeb	Water-dispersible granules						

Table 3b. Fungicides currently available for downy mildew management in outdoor and protected Onions, Peas, Parsley/Basil, and Roses/HNS.

Product Name	MAPP No.	Formulation name	Formulation type	Outdoor Onions	Protected Onions	Outdoor Peas	Protected Peas	Outdoor parsley	Protected Parsley	HNS & Roses	Protected Roses
Amistar	18039	Azoxystrobin	Suspension concentrate	✓		✓	✓			✓	✓
Azofin Plus	18552	Azoxystrobin	Suspension concentrate			✓	✓				
Amylo X WG	17978	*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747	Water-dispersible granules					✓	✓	✓	✓
Avatar	16608	Fosetyl-aluminium/propamocarb hydrochloride	Soluble concentrate							✓	✓
Captan 80 WDG	16293	Captan	Water-dispersible granules							✓	
Clayton Belfry	18154	Azoxystrobin	Suspension concentrate			✓	✓				
Conclude AZT 250SC	18440	Azoxystrobin	Suspension concentrate			✓	✓				
Folio Gold	14368	Chlorothalonil/metalaxyl-M	Suspension concentrate								✓
Fubol Gold WG	14605	Mancozeb/metalaxyl-M	Water dispersible granules	✓					✓		
Fytosave	18433	COS-OGA	Soluble concentrate					✓	✓	✓	✓
Infinito	16335	Fluopicolide/propamocarb hydrochloride	Suspension concentrate	✓	✓			✓	✓		
Invader	15223	Dimethomorph/mancozeb	Water-dispersible granules	✓				✓			
Karamate Dry Flo Neotec	14632	Mancozeb	Water-dispersible granules								
Karma	16363	Potassium hydrogen carbonate	water soluble powder					✓	✓		
Olympus	18158	Azoxystrobin/chlorothalonil	Suspension concentrate			✓	✓				
Pan Cradle	15923	Fosetyl-aluminium/propamocarb hydrochloride	Soluble concentrate								
Paraat	15445	Dimethomorph	Wettable powder					✓	✓		✓
Percos	15248	Ametoctradin/dimethomorph	Suspension concentrate							✓	✓
Plant Trust	15779	Fosetyl-aluminium	Encapsulated granule							✓	✓
Previcur Energy	15367	Fosetyl-aluminium/propamocarb hydrochloride	Soluble concentrate					✓	✓	✓	✓
Promess	16008	Propamocarb hydrochloride	Soluble concentrate							✓	✓
Proplant	15422	Propamocarb hydrochloride	Soluble concentrate							✓	✓
Revus	17443	Mandipropamid	Suspension concentrate					✓	✓	✓	✓
SL 567A	12380	Metalaxyl-M	Soluble concentrate	✓				✓	✓		
Toran	18239	Azoxystrobin	Suspension concentrate			✓	✓				
Valbon	14868	Benthiavalicarb-isopropyl/mancozeb	Water-dispersible granules							✓	✓

whereas a smaller number of fungicides is available for onion crops, with only one formulation (Infinito - Fluopicolide/propamocarb hydrochloride) registered for protected crops (Table 3b). This situation does make the formation of effective integrated control strategies both imperative and highly challenging, although there are promising new chemistries being developed internationally, for example OSBPI (*oxysterol binding protein homologue inhibition*) fungicide, Oxathiapiprolin (FRAC 49 F9), has recently been shown to have excellent systemic protectant efficacy against oomycetes including *Phytophthora infestans*, cucumber downy mildew (*Pseudoperonospora cubensis*) and *P. belbahrii* mildew of sweet basil (Cohen, 2017; 2020; Patel *et al*., 2015).

Review of downy mildew management utilising Elicitors

Introduction

The objective of this review was to collate information regarding the use of elicitors against aerial oomycete pathogens of concern to UK growers of leafy salads, onions, ornamentals (mainly downy mildews) and of tomatoes and potato (late blight).

An elicitor can be defined as a compound that induces a defence response to damage or infection in a plant, and can be biological or chemical in origin. The term biostimulant is sometimes used interchangeably with the term elicitor, however, a wider range of plant processes can be bio stimulated. There is no single, globally accepted definition of the term "plant biostimulant" for legal, regulatory, or commercial purposes, but the USA Biological Products Industry Alliance (BPIA) supports the following working definition: *Plant biostimulants contain substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality.* Biostimulants have no direct effects on diseases or pests. The AHDB website states that it should be noted that a biostimulant's main role should not be to provide fertilisation or pesticidal activity. Any product marketed as a pesticide must have a Ministerially Approved Pesticide Product (MAPP) number. Due to a lack of consensus on the term, there are no specific frameworks for regulating biostimulants in the EU, United States and other countries. The European Commission, however, intends to revise Regulation (EC) No.2003/2003 (the Fertiliser Regulation) and extend its scope to include plant biostimulants (among other materials).

Some biostimulants marketed in the UK to "promote plant health" contain likely elicitors, and these were included within a recent AHDB funded review of the function and value of biostimulant products for UK cereals and oilseeds (Storer *et al.,* 2016 - Review No. 89). An overview of the modes of action of elicitors and their potential contribution to pathogen control

was provided in the CP 157 review of management and control options in UK edible horticultural and ornamental crops (Wedgwood *et al.,* 2016). An AHDB factsheet has summarised biostimulant product types and evidence of positive effects (Awan & Storer, 2017).

The first resistance activator registered was probenazole in Japan in 1975, since then many other products have been developed such as Bion and Actigard (acibenzolar-S-methyl (ASM)). Products are still being trialled such as Reysa (*Reynoutria sachalinensis* extract) by the AHDB along with other similar products such as phosphite and salicylic acid.

It is not envisaged that all the elicitors currently sold as biostimulants will be put forward and then gain approval as plant protection products under the current regulatory framework in the UK, Recently Romeo (cerevisane) has become available as a plant protection product in the UK, as a host plant defence inducer for use on crops including tomatoes and lettuce under permanent protection with promotion mentioning downy mildew as well as powdery mildew and botrytis. FytoSave containing oligosaccharides from chitin and pectin is also now available with label recommendation against powdery mildews. It is hoped more may follow. There is grower interest in elicitors and with the loss of conventional chemistry there may before long be an accepted place for them in crop management programmes.

Materials and methods

This review examined worldwide research testing elicitors / resistance inducers against aerial oomycetes of leafy salads, onions, ornamentals (principally downy mildews), tomatoes and potatoes (blight).

One aim of the current review was to bring AHDB Research Review No. 89 by Storer *et al.,* (2016) to the attention of horticultural crop growers. Review No. 89 holds detailed information on the various types of biostimulants available to farmers and growers and their known or potential modes of action across a range of crops. This information is not duplicated here, although the factsheet produced with project findings has been included in the Appendix of the current review. Storer *et al*. (2016) produced a summary table (given in the current review) showing the evidence for positive biostimulant effects, separating out increased tolerance of pathogens as a result of the application of particular types of elicitors.

Review No. 89 produced tables of biostimulant products available in the UK and their label claims. For the current review those products claiming plant health benefits (or similar such phrases) have been extracted and placed in new tables together with details of the company supplying them, the contents (chemical and/or microbial) and the product type category. Some

products recently targeted at horticultural crops have also been added to the tables of microbial and non-microbial products, but a definitive list is not intended as new products enter the market regularly with most being different formulations of common ingredients.

Information on elicitors tested against aerial oomycetes was sourced from refereed and un-refereed research using the Google Scholar, Web of Science and Google search engines for aerial oomycetes determined to be of economic importance (utilising information from the AHDB industry survey of CP 157 in Wedgwood *et al.,* 2016) (Table 4 & Table 5). Searches were carried out utilising the Latin name of these pathogens together with the host common name and the term elicitor. Where possible, the level/s of control achieved and the conditions in which these results were obtained were extracted, because responses to the application of elicitors has been reported to vary much more than for fungicides owing to the fact that they operate indirectly by stimulating the host to increase its own defences against the invading pathogen. Information has been tabulated to aid comparison between products. Products registered as biofungicides such as Serenade ASO (*Bacillus subtilis*) and Prestop (*Gliocladium catenulatum*) available against root infecting oomycetes and products such as herbal oils have not been included in these tables as they are known to principally work directly to cause pathogen mortality, although they can also elicit host defence responses to reduce tissue invasion.

Another review, of research carried out between 1973 and 2008, produced by the European Union (ENDURE, 2011) resulted in comprehensive tables of references separating success in field trials from success in laboratory conditions by microbial, botanical and other agents against *Phytophthora infestans* potato and tomato blight, and *Bremia lactucae* lettuce downy mildew. These have been extracted directly into the current report, together with a table for grape downy mildew *Plasmopara viticola* detailing further products shown to have benefit. Further details of the work were sought for the botanical and “others” product categories to add to the disease level control tables if the references had not already been located using search engines.

In addition, general reviews of elicitors were sought and have been referenced to direct readers to information on their use against pathogens other than oomycetes and on crops outside the scope of the current short review. These reviews include information on the biochemistry and host genetics considered to be at work in plants’ defence mechanisms and by extrapolation how externally applied elicitors may work. These should be accessed to find out about the activity of individual product types, however information on the general principles of systemic acquired resistance has been incorporated in the current review from AHDB CP 157 (Wedgwood et al., 2016) to aid understanding of the principles of elicitor activity.

Table 4. Edible crops: Late blight of tomato and potato and downy mildews of onion and leafy salads of concern to UK growers, with Latin names

Latin name of crop	Crop common name	Latin name of pathogen	Pathogen common name
Solanum tuberosum	Potato	*Phytophthora infestans*	Late blight
Solanum lycopersicum (was *Lycopersicon esculentum*)	Tomato	*Phytophthora infestans*	Late blight
Allium cepa	Onion	*Peronospora destructor*	Downy mildew
Allium schoenoprasum	Chives	*Peronospora destructor*	Downy mildew
Brassica spp. including *rapa, juncea, narinosa*	as Baby Leaf, and Chinese Leaf	*Hyaloperonospora parasitica (Peronospora parasitica)*	Downy mildew
Lactuca sativa	various Lettuce types	*Bremia lactucae*	Downy mildew
Spinacia oleracea	Spinach	*Peronospora farinosa* f.sp. *spinaciae*	Downy mildew
Eruca vesicaria and *Diplotaxis tenuifolia*	Rocket – salad and wild	*Hyaloperonospora parasitica (Peronospora parasitica)*	Downy mildew
Nasturtium officinale (Rorippa nasturtium-aquaticum) a Brassica	Watercress	*Hyaloperonospora parasitica (Peronospora parasitica)*	Downy mildew
Cichorium spp.	Radicchio & Endive	*Bremia lactucae*	Downy mildew
Beta vulgaris subsp. *vulgaris*	Chard	*Peronospora farinosa* f.sp. *betae*	Downy mildew
Beta vulgaris	Red Beet as Baby Leaf	*Peronospora farinosa* f.sp. *betae*	Downy mildew
Ocimum basilicum	Basil as Baby Leaf Herb	*Peronospora belbahrii*	Downy mildew
Anthriscus cerefolium	Chervil as Baby Leaf Herb		Downy mildew
Coriandrum sativum	Coriander as Baby Leaf Herb	-	None noted

Table 5. Ornamental plants: Downy mildews and aerial *Phytophthora spp.* of nursery stock, pot, bedding and cut flowers of concern to UK growers, with Latin names

Latin name of crop	Common name of crop	Latin name of pathogen	Common name of pathogen
Various including *Rhododendron ponticum, Larix decidua & kaempferi, Quercus rubra, Vaccinium myrtillus*	Rhododendron, Larch, Oak, Bilberry and others	*Phytophthora ramorum* & *Phytophthora kernoviae*	Phytophthora (Sudden oak death)
Antirrhinum spp.	Antirrhinum (including Snap Dragon)	*Peronospora antirrhini*	Downy mildew
Aquilegia vulgaris	Aquilegia (Columbine)	*Peronospora* sp.	Downy mildew
Hebe spp.	Hebe	*Peronospora grisea*	Downy mildew
Impatiens walleriana	Impatiens (Bizzy Lizzy)	*Plasmopara obducens*	Downy mildew
Eustoma grandiflorum	Lisianthus	*Peronospora chlorae*	Downy mildew
Nicotiana spp.	Nicotiana	*Peronospora hyoscami* f.sp. *tabacina*	Downy mildew
Papaver somniferum & *Meconopsis betonicifolia*	Poppies	*Papaver somniferum*	Downy mildew
Viola spp.	Pansy & Viola	*Peronospora violae*	Downy mildew
Rosa spp.	Rose	*Peronospora sparsa*	Downy mildew
Senecio cineraria (*Jacobaea maritima*)	Senecio	*Pustula* sp.	White Blister

Results

Biostimulant products and elicitors

There is a wide range of biostimulants; acids, microbials, extracts and others (Figure 1) and some have been show to elicit plant defence responses to fungi and/or oomycetes.

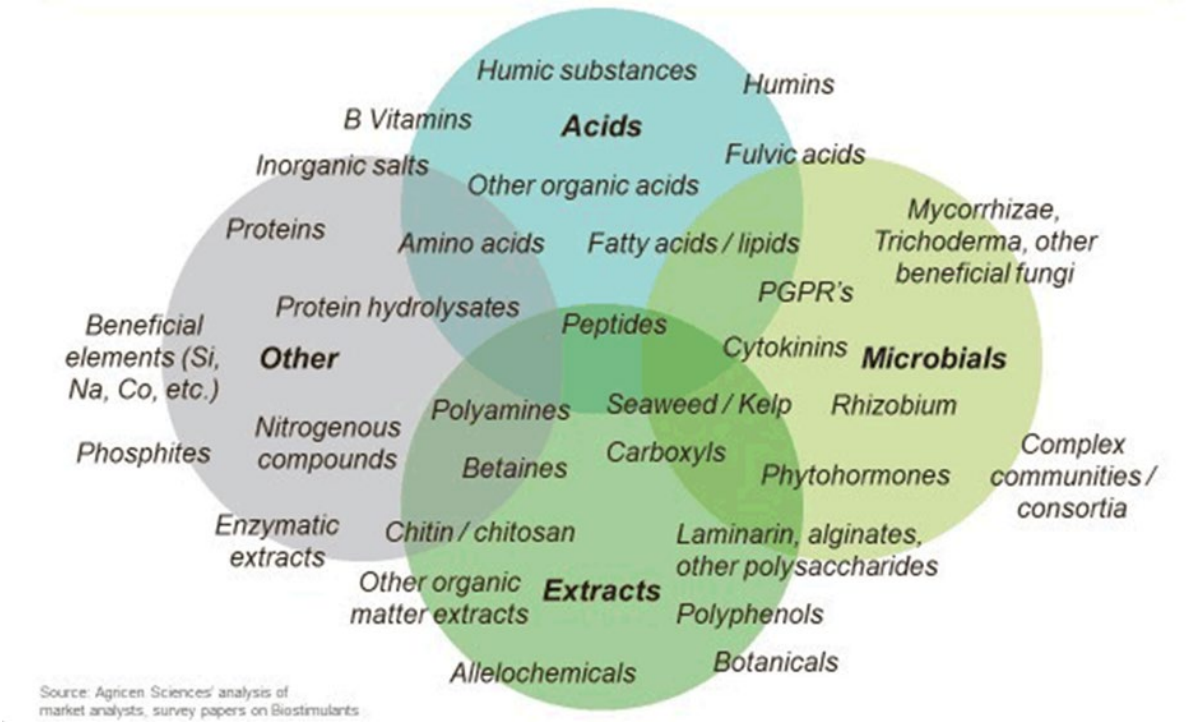

Figure 1. Biostimulant grouping. Sourced from the USA Biological Products Industry Alliance website https://www.bpia.org/solutions-provided-by-biological-products-biostimulants

Evidence for beneficial effects of different types of biostimulants on cereals and oilseed rape was summarised as a table by Storer *et al*., (2016) and this is reproduced here (Table 6). Many of the studies reviewed were principally carried out in the laboratory, rather than in normal crop situations. The review found some product types caused induced or physical resistance or tolerance to pathogens, these were: seaweed extracts, phosphite and organic salts chitin and chitosan derivatives, non-essential chemical elements, plant growth promoting bacteria, non-pathogenic fungi and arbuscular mycorrhizal fungi. The nature of these products and the various modes of action known, or hypothesised, was covered in full by Storer *et al.* (2016) and so is not repeated here.

Table 6. Biostimulant product types and positive effects. The level of evidence (* low to *** high) for cereals and oilseed rape. Biotic stress tolerance †† induced or physical to pathogen

Effect Category	Nutrient uptake or access			Plant function & Growth						Abiotic stress tolerance				Biotic stress tolerance	
Product type	N	P	Other	Hormonal	Growth†	Yield	Reduced Transpiration	Delay senescence	Improved photosynthesis	Salt	Alkaline	Drought	Cold	Pathogen††	Pest††
Seaweed extracts	*	*	*	**	**	**			*	*		*	*	*	*
Humic substances	**	*	*	*	**	**			*	*		*			
Phosphite & inorganic salts				*	**	**								**	
Chitin & chitosan derivatives					**	**	*			*		*	*	***	*
Anti-transpirants				***		**a	***		*			**			
Protein hydrolysates & amino acids	*		*		*	*				*		*	*		
Non-essential chemical elements	*	*			*	*		*	*	*	*	*		**	**
Plant growth promoting bacteria	**	**	*	*	***	***				*		*		**	*
Non-pathogenic fungi	*	*	*	*	**	**				*		*	*	**	
Arbuscular mycorrhizal fungi	*	**	*		**	**						*		*	*
Protozoa & nematodes	*			*	*	*									

Other reviews across a wider range of crops have been carried out and provide further references showing efficacy against disease or sometimes otherwise (Burketova *et al*., 2015; Hadwiger, 2013; Tamm *et al*., 2011; Walters *et al.,* 2013). These have concentrated on chemicals and commercial products that have been tested as elicitors of plant defence against pathogens with, in general, the term “fungi” taken to include the oomycetes (with cellulose walls) as well as chitin / chitinase-walled fungi. Cellulose, chitin and cellulose are all β-1,4-polymers of glucose (Hadwiger, 2013) and so if invading mycelium identification is utilised in plant defence reactions, then the reaction stimulated by products reported acting against a broad spectrum of fungi is likely to act against oomycetes similarly.

Modes of action of elicitors

When a plant experiences disease (or pest) attack various defence responses are induced to enhance their resistance. Signal inducing compounds (elicitors) are perceived by the plant’s innate immune system that prime and/or elicit plant defence responses (Weisel *et al.,* 2014). The induction of plant resistance to biotic challenges can lead to the direct activation of defences, but can also lead to the priming of cells, resulting in stronger elicitation of those defences following pathogen attack (Goellner & Conrath, 2008). Treatment of plants with various agents, including cell wall fragments, plant extracts and synthetic chemicals can induce resistance to pathogen attack both locally and systemically. Elicitation does not necessarily result in complete control of pathogens, but can for example lead to reduced lesion size and/or number (Sillero *et al.*, 2012; Walters *et al.*, 2013).

Modes of action differ between different types of elicitor (Figure 2) (Henry *et al.,* (2012). Pathogen-Associated Molecular Patterns (PAMPs) trigger signals as a result of pathogen attack on the plant. Wounding by mechanical handling, pathogen or insect attack may trigger

Damage Associated Molecular Patterns (DAMPs). Signalling after application of agents or non-pathogenic microbes comes from Microbe-Associated Molecular Patterns (MAMPs).

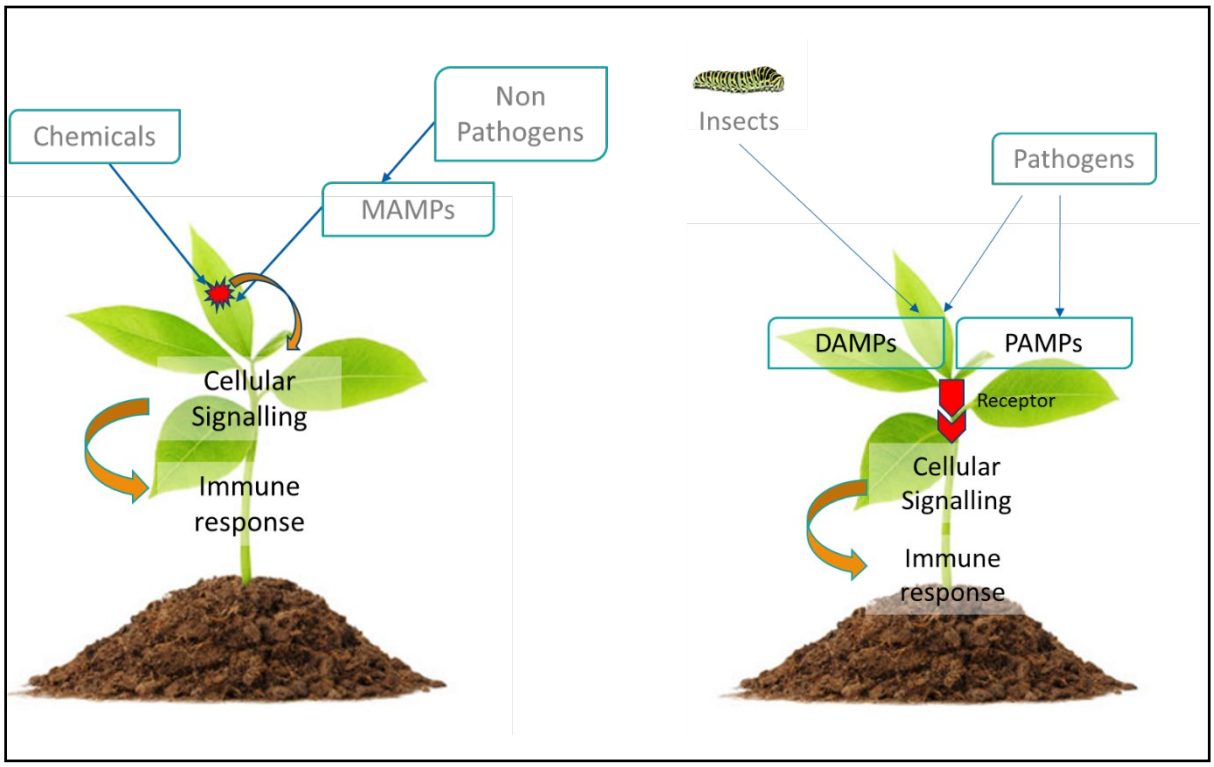

Figure 2. Systemic defence responses by plants (derived from Henry *et al*., 2012).

A number of different types of induced resistance have been defined based on differences in signalling pathways and spectra of effectiveness including systemic acquired resistance (SAR) triggered upon pathogen attack, foliar treatments of plants with MAMPs, DAMPs, plant phytohormones or chemical activators and induced systemic resistance (ISR) which can be triggered by root colonisation with plant-growth-promoting rhizobacteria (PGPR) or fungi (Burketova *et al.*, 2015). Such resistance can be induced in plants by application of a variety of biotic and abiotic agents. The resulting resistance tends to be broad-spectrum and can be long-lasting, but is rarely complete, with a review concluding that most inducing agents reducing disease by between 20 and 85% (Walters *et al*., 2013).

Immune responses are orchestrated by and depend on phytohormones such as salicyclic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) (Burketova *et al*., (2015). SAR is induced by treatment with a variety of agents (such as Bion, acibenzolar-S-methyl (ASM), beta-amino-butyric acid (BABA) and cis-jasmone) and is mediated by a salicylic acid-dependent process. ISR is mediated by a jasmonate and ethylene-sensitive pathway (Walters *et al.*, 2013). Extracts from *Ulva armoricana* give high elicitor activity from their complex sulphated hetero-polysaccharides (Jaulneau *et al.*, 2011; Walters *et al.*, 2013).

After the recognition of the attack by a sensor, the plant cell triggers complex signalling and defence responses including the production of pathogenesis-related proteins, antimicrobial phytoalexins or cell wall fortification with callose or lignin. Local defences are followed by the production of mobile signals that are transported via the xylem to prime distal plant parts for accumulation of defence compounds (Burketova *et al.*, 2015).

Types of elicitors

Elicitors fall into four main types; chemical/synthetic, microbial derived (fungal & bacterial), plant extracts/components and algal extracts. Detailed information on the types and activity of biologically based elicitors is given in the review by Burketova et al. (2015), with a more exhaustive review of chemical elicitors made by Walters *et al.* (2013). Reviews by Da Rocha & Hammerschmidt (2005) and Thakur & Sohal (2013) have tabulated the effects on a range of crop species of trials of various elicitors. The effects of elicitors at the molecular level are given by Weisel *et al*., (2014). Reference should be made to these reviews for details of the biochemical activity of the elicitors and extensive reference listings of disease control experiments. A summary of the most commonly tested elicitors and an indication of their activity is given in Table 7.

Table 7. Types of elicitor and examples of each with any product names, and the types of resistance and the nature of resistance.

Elicitor type	Name & commonly used abbreviation	Example products or source	Nature of resistance* & pathogen/host noted
Chemicals			
	acibenzolar-S-methyl (ASM) = benzothiadiazole (BTH)	Bion Actigard	Broad-spectrum of hosts & pathogens, SA-activated SAR
	β-amino butyric acid (BABA)	-	Broad-spectrum SAR, (also some fungitoxicity)
	Probenazole and its metabolite Saccharin	Oryzemate	SAR. Mainly used on rice against a fungus & bacterium
	Potassium phosphite N.B. recently registered as a pesticide in EU	HortiPhyte Farm-Fos TKO Phosphite	Stimulation via SA & JA/ET pathways against oomycetes. Direct inhibition of *Phytophthora* spp. growth
	Silicon compounds (silicates, stabilised silicic acid & silica nanoparticles)	Sion	Repeated silicate application reduces powdery mildews. Silicic acid reduces bacterial & fungal infections
Microbial derived			
Bacterial	Curdlan (glycopentaose)	*Agrobacterium* sp.,	*P. infestans* on potato

Elicitor type	Name & commonly used abbreviation	Example products or source	Nature of resistance* & pathogen/host noted
	2,4-diacetylphloroglucinol	*Pseudomonas fluorescent* (PGPR).	ISR against *Peronospora parasitica* on Arabidopsis
	HrpN (harpin)	*Erwinia amylovora*	HR, SAR against *Peronospora parasitica* on Arabidopsis
Fungal	Chitin & chitosan	ex Fungal cell walls, crustacean shells, algae	Against fungi, eliciting plant cell changes including DNA alteration, phytoalexins, lignification & callose deposition
	Poly- & oligoglucans	β-1,4-glucans ex plant cellulose	
Plant extracts			
Algal	Laminarins, β-1,3-glucans storage polysaccharides	Brown algae e.g. *Laminaria digitata*	Elicitor of defence responses & inducer of resistance against *Plasmopara viticola* in grapevine, fungi & virus
	Ulvans, heteropolysaccharides	Green algae of *Ulva* genus	Broad range of defence mechanisms, with jasmonic acid signalling & phytoalexins
	Carrageenans, linear polysaccharides	Red algae	Induce resistance against a broad range of pathogens
	Fucans	Brown algae *Ascophyllum nodosum, Fucus* spp. & *Ecklonia* spp.	Local & systemic resistance to tobacco mosaic virus
Higher plants	Herbal extracts, a mixture of diverse compounds	Various	Direct antimicrobial effects. Constituents likely influence plant metabolism synergistically either directly or by priming.
	Giant knotweed extract	Milsana *Fallopia (Reynoutria) sachalinensis*	Anti-microbial. Defence induced peroxidase activity, phytoalexin concentration against fungi.
	(Neem) a pesticide	*Azadirachta indica*	Anti-microbial and resistance-inducing against fungi & bacteria
	Essential oil, containing methyl salicylate	*Gaultheria procumbens*	Salicylic acid defence response
Composts	Efficacy likely from the microbial populations		Compost-induced resistance similar to both SAR and ABA-dependent/independent abiotic stress responses
Biochar	Coproduct of pyrolised biomass		Systemic resistance including against *Phytophthora cinnamomi* & *Phytophthora cactorum* in oak and maple seedlings

* Broad-spectrum resistance includes effects having been shown in experiments against bacteria, fungi (including oomycetes) and viruses. ABA abscisic acid; SA, salicyclic acid; SAR, Systemic acquired resistance; ISR, Induced systemic resistance; HR hyper-sensitive response.

Phosphorous acid (potassium phosphite/phosphonate) is an elicitor previously included in HDC/AHDB Horticulture plant disease control trials, as HortiPhyte, Farm-Fos or TKO Phosphite 0-29-26. It is the anionic metabolite of the systemic fungicide aluminium tris-O-ethyl phosphonate (fosetyl-Al), effective against oomycetes. With wetter TKO Phosphite gave equivalent control to fungicides at low-moderate downy mildew pressure on roses, but was less effective at high pressure (O'Neill, 2007). Control was also reported in a further project (O'Neill, 2014). Control of tomato *Phytophthora* blight has also been shown by this chemical (Becktell, 2005). Phosphorous acid had a limited effect in reducing the area of *P. infestans* on potato leaves, but its systemic effect was reported as reducing the incidence and severity of tuber rot caused by the pathogen (Johnson *et al.,* 2004). As potassium phosphite has recently been registered in the EU as a fungicide (Bolette Palle-Neve, AHDB pers. comm. January 2020) it has been excluded from further mention in this report concentrating on products that produce their effects through the plant without direct effect on pathogens.

Burketova *et al.* (2015) listed 31 bacteria-derived compounds (BDCs) originating from one or other of at least a dozen species, they are either secreted by the bacteria, part of the outer shell, or abundant after being released accidentally from bacteria. Table 7 gives BDCs that have been effective against certain oomycetes. The use of silicon compounds as foliar sprays on crops to increase growth and decrease biotic and abiotic stresses was reviewed by Laane (2018) with reduction of powdery mildew in particular by silicate, but with no reported tests on downy mildews or other oomycetes. Their modes of action are not fully elucidated, although elicitor action is hypothesised.

Chitosan source, chemistry and its use in plant defence has been reviewed by Hadwiger (2013) and he has proposed a scheme describing chitosan generation, signalling routes and mechanisms of defence gene activation. However, none of the research quoted was carried out on oomycetes. In the USA, chitosan as the product Elexa provided moderate control of downy and powdery mildew (*Plasmopara viticola* and *Uncinula necator)* in a vineyard (Schilder *et al.,* 2002). Chitosan, best at lower chitosan concentrations of 10 to 50 µg a.i. per plant, was reported to reduced strawberry crown rot *Phytophthora cactorum* symptoms (Eikemo *et al.,* 2003).

Laminarin is a storage polysaccharide of the Kelp seaweed *Laminaria digitata*. Grapevines challenged with downy mildew, *Plasmopara viticola,* regulated the expression of genes which encode chitinases, and it was shown that laminarin treatment of grapevine cells increased the expression of these genes (Minami, 2004).

Salicylic acid, chemical, found in aspirin, has been used to induce host resistance in several plant species. Applied as a spray at 0.2 g/L, it was reported to significantly reduce the leaf area affected by downy mildew of lettuce *Bremia lactucae* (DEFRA, 2002).

In addition to non-living elicitors, microbes can be applied to roots and result in induced systemic resistance (ISR). These include plant-growth promoting rhizobacteria (PGPRS) such as *Pseudomonas fluorescens*, biofungicides such as various *Trichoderma* species and arbuscular mycorrhizal fungi. These are reviewed by Walters *et. al.* (2013), with infection by various foliar and root infecting fungi having been shown to be reduced, but no examples are given specifically for oomycetes.

Plant defence responses have a genetic basis and the DRASTIC (Database Resource for the Analysis of Signal Transduction in Cells) gene expression database (currently manually curated by Gary Lyon) was set up in 2001 as a collaborative project between staff at the Scottish Crop Research Institute (SCRI) and the University of Abertay, Dundee. The DRASTIC web site https://www.drastic.org.uk is a database of plant-expressed sequence tags and genes up- or down-regulated in response to various pathogens (biotic stress), chemical treatments, and abiotic stress such as drought, salt and cold. The database currently contains over 33,400 records and includes information from a wide range of plant species including the "geneticists model plant" *Arabidopsis thaliana*, tomato (*Lycopersicom esculentum*) and potato (*Solanum tuberosum*). There is a road map facility to enable the creation of look-up tables to find genes that are co-regulated by treatments. While primarily intended for use by researchers in plant breeding, understanding what genes are involved in defence responses could help with the selection of types of elicitors and gain an indication of whether then could be effective on different hosts.

In the current project the crops reviewed were mostly tested in glasshouses and as most are grown commercially under protection (for some time at least, other than for potato) any effect reported is likely to be reproducible in a crop. As true for much published research on any topic it is likely that experiments that failed to show responses are under-represented in the literature. Much of the research has been carried out with single chemicals in order to see their effects, rather than on products. This has the advantage that an indication of the activity of different products can be guided by examining the ingredients of the particular product available to a grower. An indication is only possible as the nature/strength of any activity may differ at different concentrations of the elicitor and there are likely to be effects on plants from the other chemicals or microbes in the product.

Storer *et al*., (2016) tabulated the biostimulant products available to UK cereals and oilseeds growers, detailing the contents and the product aims as given on the label. Those with label claims of improving resistance to disease or reinforcing plant defence have been abstracted from Storer *et al*., (2016) for this report (Tables 8, 9 & 10). Some contained microbes such as root-colonising bacteria (rhizobacteria) or arbuscular mycorrhizal fungi, or non-microbial products including seaweed / algae extracts (mainly *Ascophyllum nodosum*), L-amino acids, silicon, chitosan, phosphite, humic and fulvic acids, humic-lignate, bioflavonoids, fruit acids and fatty acids or both. Details of the substances and their probable modes of action were reviewed in detail by Storer *at al.* (2016) and reference should be made to this report, including the conclusion section where knowledge gaps were highlighted. Terms relevant to biostimulants are given in the Glossary.

These and similar products are available to horticultural crop growers and sports turf maintainers and a wide range are promoted at trade shows, via the trade press and web sites. Examples of suppliers and currently available products can be found using a search engine for “UK plant biostimulant suppliers”. This review concentrates on the potential plant defence activation properties of individual components as established via independent, replicated experiments. Concentrating on the active ingredients rather than the changing market of specific products, and any manufacturer’s claims for their formulations.

Table 8. Microbial plant health stimulants.

Product	Company	Target Crop	Product Contents	Product aim (as described on label)	Product type category	Application type
RGPRO AgGrow 3	PlantWorks Ltd.	Oilseed Rape	Plant growth promoting rhizobacteria at CFU/MI 10^8 : *Gluconacetobacter diazotrophicus*, *Agrobacterium* spp., *Bacillus amyloliquefaciens*, *Bacillus megaterium*, *Azosprillum brasilens*, *Rhizobium* spp.	Improves nutrient uptake, plant health and development and stress tolerance. Improves soils health and biological status	PGPR	Granular soil application, seed drilling or broadcast
Symbio liquid endo mycorrhizal inoculant	Symbio	Cereals	Arbuscular mycorrhizal fungal inoculant	Increases plant growth in poor soils, increases yield, healthy plants are more resistant to stress and disease, reduces need for fertiliser and water	Arbuscular mycorrhizal	Seed coat, soil drench or mixed with compost teas

Table 9. Non-microbial products containing a single ingredient.

Product	Company	Target Crop	Product Contents	Product aim (as described on label)	Product type category	Application type
ALGAFlex	Biotechnica	Cereals & Oilseed Rape	Concentrated seaweed extract, principally derived from *Ascophyllum nodosum*	Increase yield, strengthen root system development. Improve tolerance to environmental stresses and diseases and increase activity of beneficial microbes	Seaweed extract	Soil drench or foliar spray
BlaminoAM3	Biotechnica	Cereals & Oilseed Rape	L-amino acids	Strong and sustainable vegetative growth, increase crop yield and quality, improve resistance to environmental stresses, enhanced disease resistance	Amino acids	Foliar spray
BioSilicate	Biotechnica	Cereals	Biologically available silicon	Stronger stalks and stems, reducing lodging, better photosynthesis from extended leaves and extra chlorophyll, resistance to fungal pathogens and sucking insects such as aphids, improved resistance to high salts or toxins	Non-essential chemical elements	Foliar spray
Symbio Chitogro	Symbio	Cereals & Oilseed Rape	Chitosan	Stimulates healthy root growth , increases recovery rate after pathogen attack, improves germination and seedling survival rates, stimulates beneficial soil biology	Chitosan	Foliar spray

Table 10. Non-microbial products containing multiple active ingredients.

Product	Company	Target Crop	Product Contents	Product aim (as described on label)	Product type category	Application type
C Weed 50	Micromix	Cereals and Oilseed Rape	50% w/v seaweed concentrate produced at lower temperatures utilising only *Ascophyllum nodosum* – formulated with Humic acids and harvested only during selected periods of growth	Earlier establishment, increases early rooting , photosynthetic area, leaf and shoot growth and plant carbohydrate production, improves sugar content in treated crops, resistance to disease and pests, storability of treated crops, improves shelf-life of plants and flowers	Seaweed extract	-
C-Weed AAA	Micromix	Cereals and Oilseed Rape	*A. nodosum* concentrate produced from cool extraction process, plus a wide range of L-amino acids from fermentation of plant extracts	Earlier establishment, increases early rooting, photosynthetic area, leaf and shoot growth, plant carbohydrate production, improves sugar content in treated crops, improves resistance to disease and pests, storability of treated crops	Seaweed extract with L-amino acids	-
Optiphite GP	Micromix	Cereals and Oilseed Rape	Nitrogen, Phosphite, Potassium, Phosphate, amino acids, humate-lignate active-uptake formulation technology	Reinforces plant disease defence and enhances root development	Phosphite, amino acids, humic substances	-
Patron Z	Micromix	Cereals and Oilseed Rape	Nitrogen, Zinc, Ammonium Acetates, Amino acids (wide range) with alkyl polyglucoside surfactant (with humic acids)	Enhances root and seedling development, increases root mass and length, improves seedling disease resistance, improves nutrient uptake efficiency	Amino acids, humic acids	-
VitAmix	Micromix	Cereals and Oilseed Rape	Potassium, phosphite, humic and fulvic acids, chelated copper, Manganese, Zinc, + Boron, Molybdenum	Improves seedling establishment , promotes root development, reduces disease, corrects deficiencies and prevents physiological disorders	Phosphite, humic substances	-

Product	Company	Target Crop	Product Contents	Product aim (as described on label)	Product type category	Application type
AMIX Micronutrients	Micromix	Cereals and OSR	Humic-lignate complexed Copper, Manganese, Zinc, Iron, Calcium, Magnesium and combinations	The AMIX range are all biostimulants and all produce yield increases in the absence of deficiency and are capable of increasing plant health levels	Humic substances with non-essential chemical elements	-
Bio 20	OMEX	Cereals and OSR	Biostimulant, N,P,K	Fertiliser, stress relief and plant health promotion	Seaweed extracts	Foliar spray
ProAlexin PNS and ProAlexin PEL	Micromix / Phyto Innovation Ltd	Cereals and OSR	Blend of citrus bioflavonoids, fruit acids (Citric acid, Lactic acid, Malic acid), Essential Fatty Acids (Caprylic Acid), Palm Kernel Oil Extract	Synergistic blend promotes health and survival, improves speed of growth, yield and crop quality	Other-Citrus extracts, Natural Acids	-
Sentinel	Engage Agro Europe	Cereals and OSR	Silicon w/w + salicylic acid	Protection against biotic stress using the silicon to increase the cell wall strength. The salicyclic acid supports wound repairing reducing sites for disease ingress.	Micronutrient	Foliar spray
Sinergy	Micromix	Cereals and OSR	An NPK liquid based on Phosphite with Silicon and amino acids	Improve quality and plant health	Phosphite, non-essential chemical elements and amino acids	-
Vitomex	OMEX	Cereals and OSR	Phosphite (PO_3) with Potassium, Magnesium, Copper and Zinc	Improves plant health and tolerance of abiotic stress	Phosphite	Foliar spray

Activity of elicitors against downy mildews and late blight

The ENDURE project (European Network for Durable Exploitation of crop protection strategies) reviewed literature sources between 1973 and 2008 for biocontrol agents shown to be successful against downy mildew and/or late blight in either the field, laboratory or growth cabinets. The tables from Appendix 13 are given here for potato blight (Table 11a), tomato blight (Table 11b), vegetables, fruits and ornamentals (Table 11c). Grapevine (Table 11d) is included because information for other downy mildews is sparse. The majority of the fungal or bacterial organisms, plant extracts/botanicals and other compounds are not marketed within plant protection products in Europe. All product activity types are shown in the table including direct activity by microbes used in fungicides, but induction of a host defence response is also known for them. Expression of a response by a plant to disease under field conditions is likely to be influenced by a number of factors, including the environment, genotype, crop nutrition and the extent to which plants are already induced. Although research in this area has increased over the last few years, our understanding of the impact of these influences on the expression of systemic acquired and induced resistance is still poor. There have also been a number of studies in recent years aimed at understanding of how best to use defence elicitors in practical crop protection. However, a recent review concluded that such studies are relatively rare and further research geared towards incorporating induced resistance into disease management programmes, if appropriate, is required (Walters *et al*., 2013).

Tables 11a-d. Inventory from ENDURE of biocontrol agents (M: microbial; B: botanicals; O: others) described in primary literature (1973-2008) for successful effect against downy mildew / late blight pathogens in laboratory experiments and field trials on selected crops.

Potato (target pathogen = *Phytophthora infestans*)		
	Success in field trials	**Success in laboratory conditions** (*in vitro* and/or *in planta* in controlled conditions)
M	*Bacillus subtilis* (Basu et al., 2001) *Bacillus* sp. isolate PB2 (Atia, 2005) effect < fungicides *Pseudomonas fluorescens* (Basu et al., 2001) *Pseudomonas fluorescens* isolate PPfl (Atia, 2005) effect < fungicides *Pseudomonas* (El-Sheikh et al., 2002) *Gliocladium virens* (Basu et al., 2001) *Phytophthora cryptogea* (Quintanilla, 2002) *Trichoderma* spp (Saikia and Azad, 1999) *Trichoderma viride* (Basu et al., 2001) (Basu and Srikanta, 2003) but no effect in other studies (Singh et al., 2001) (Arora et al., 2000) (Arora et al., 2006) **little or no effect once in the field (good in lab):** *Acremonium strictum, Penicillium viridicatum* and *Penicillium aurantiogriseum* (Arora, 2000) (Arora et al., 2006) *Myrothecium verrucaria* and *Chaetomium brasiliense* (Arora et al., 2006)	Serenade (*Bacillus subtilis* strain QST 713) (Stephan et al., 2005) (Olanya and Larkin, 2006) *Bacillus subtilis* B5 (Ajay and Sunaina, 2005) *Bacillus, Pseudomonas, Rahnella*, and *Serratia* (Daayf et al., 2003) *Enterobacter cloacae* (Slininger et al., 2007) *Pseudomonas fluorescens* (Slininger et al., 2007) *Xenorhabdus bovienii* (Eibel et al., 2004) *Penicillium aurantiogriseum* (Jindal et al., 1988) *Penicillium viridicatum* (Hemant et al., 2004) Trichodex (Stephan et al., 2005) *Trichoderma viride* (Hemant et al., 2004) *Penicillium, Rhizoctonia* and *Trichoderma* spp (Phukan and Baruah, 1991) various microorganisms (Stephan and Koch, 2002)
B	carvone (Quintanilla, 2002)	carvone , thymol, pinochamphone, plumbagin (Quintanilla, 2002) extracts of *Rheum rhabarbarum* and *Solidago canadensis* (Stephan et al., 2005) oregano extract (Olanya and Larkin, 2006) Elot-Vis (Stephan et al., 2005) patatin J from potato tuber (Sharma et al., 2004)
O	culture filtrates from Streptomyces padanus (Huang et al., 2007) negative effect: salicylic acid (Quintanilla, 2002)	chitosan ElexaTM (Acar et al., 2008) cyclic lipopeptides from Pseudomonas: massetolide A (Tran Thi Thu, 2007) extracts from *Pseudomonas fluorescens* (Martinez and Osorio, 2007)

Table 11b.

Tomato (target pathogen = *Phytophthora infestans*)		
	Success in field trials	**Success in laboratory conditions** (*in vitro* and/or *in planta* in controlled conditions)
M	*Bacillus cereus* (Silva et al., 2004) *Burkholderia* (Lozoya-Saldana et al., 2006), *Pseudomonas* (Lozoya-Saldana et al., 2006), *Streptomyces* (Lozoya-Saldana et al., 2006)	*Bacillus pumilus* (Yan et al., 2002) *Cellulomonas flavigena* (Lourenco Junior et al., 2006) *Pseudomonas fluorescens* (Yan et al., 2002) (Ha et al., 2007) (Tran Thi Thu, 2007) *Streptomyces* sp. AMG-P1 (Lee et al., 2005) *Aspergillus* sp., (Lourenco Junior et al., 2006) *Candida* sp. (Lourenco Junior et al., 2006) *Cryptococcus* sp. (Lourenco Junior et al., 2006) *Fusarium oxysporum* (Kim et al., 2007a) *Penicillium* sp. (Perez Mancia and Sanchez Garita, 2000) *Trichoderma harzianum* T39 (Ferrari et al., 2007)
B	Nochi leaf extract (Vanitha and Ramachandram, 1999)	capsidiol (El-Wazeri and El-Sayed, 1977) Elot-vis (Ferrari et al., 2007)
O	compost extracts (Zaller, 2006)	acibenzolar-S-methyl (Becktell et al., 2005) beta -amino butyric acid (Yan et al., 2002) Bion (benzothiadiazole) (Surviliene et al., 2003) bikaverin and fusaric acid (Son et al., 2008) cellulose (Perez Mancia and Sanchez Garita, 2000) chaetoviridin A (Park et al., 2005a) chitosan ElexaTM (Acar et al., 2008) Chitoplant (Ferrari et al., 2007) extracts from actinomycete isolates (Mutitu et al., 2008) extracts from *Bazzania trilobata* and *Diplophyllum albicans* (Tadesse et al., 2003) extract from *Gibberella zeae* (Kim et al., 1995) phosphate (Becktell et al., 2005)

Table 11c.

Other Vegetables and fruits

	Success in field trials	**Success in laboratory conditions** (*in vitro* and/or *in planta* in controlled conditions)
Cauliflower and other crucifers (target pathogen = *Peronospora parasitica*)		
M		Pseudomonas sp. XBC-PS (Li et al., 2007) Trichoderma harzianum (Pratibha et al., 2004)
B		
O	Bion (Pratibha et al., 2004) phosphonate (Kofoet and Fischer, 2007)	Bion (Gawande and Sharma, 2003)
Lettuce (*Bremia lactucae*)		
M		
B		
O	phosphonate (Kofoet and Fischer, 2007) Trichodermin (Borovko, 2005) Pimonex, Timorex and also Alkalin potassium+silicon (Robak and Ostrowska, 2006)	
Melon / cucumber (target pathogen = *Pseudoperonospora cubensis*)		
M		actinomycete (Shu and An, 2004) Bacillus strains, Z-X-3 and Z-X-10 (Li et al., 2003)
B		
O	phosphonate (Kofoet and Fischer, 2007)	attenuated cucumber mosaic cucumovirus (Qin et al., 1992) chitosan ElexaTM (Acar et al., 2008) compost extracts (Winterscheidt et al., 1990)

Miscellaneous

M	Azotobacter slight effect against Peronospora arborescens on opium poppy (Chakrabarti and Yadav, 1991)	Cladosporium chlorocephalum against Peronospora arborescens (Chaurasia and Dayal, 1985) (Nalini and Rai, 1988)
B		
O	phosphonate against Peronospora destructor on Allium (Kofoet and Fischer, 2007)	DL- beta -amino-n-butyric acid (BABA) against Plasmopara helianthi (Tosi and Zazzerini, 2000)

Table 11d.

Grapes (target pathogen = *Plasmopara viticola*)		
	Success in field trials	**Success in laboratory conditions** (*in vitro* and/or *in planta* in controlled conditions)
M	Bacillus brevis (Schmitt et al., 2002) Bacillus subtilis (Serenade) (Schilder et al., 2002) Pseudomonas fluorescens (Rizoplan) (Kilimnik and Samoilov, 2000) (Rajeswari et al., 2008) Fusarium proliferatum (Falk et al., 1996) Trichoderma harzianum T39 (Vecchione et al., 2007) **little or no effect once in the field:** Bacillus licheniformis (Cravero et al., 2000) Biorange (Bacillus subtilis, Candida oleophila, Pseudomonas spp. and Streptomyces spp.) (Spera et al., 2003)	Alternaria alternata (Musetti et al., 2004) Fusarium proliferatum (Bakshi et al., 2001)
B	Croplife (citrus and coconut extract) (Schilder et al., 2002) Plantfood (foliar fertilizer) (Schilder et al., 2002) Milsana (giant knotweed extract) (Schilder et al., 2002) (Schmitt et al., 2002) neem (Rajeswari et al., 2008)	neem (Achimu and Schlosser, 1992) extract of giant knotweed (Schmitt, 1996)
O	acylbenzolar-s methyl (Dagostin et al., 2006) chitosan (Elexa) (Schilder et al., 2002) Mycosin (Angeli et al., 2006)	Alternaria alternata extracts (Musetti et al., 2006) EXP1, copper gluconate, salt of fatty acid, plant based alcohol extract (Dagostin et al., 2006)

Further literature searches have shown that Sharma *et al*., (2010) found that BABA provided almost complete control of late blight (*Phytophthora infestans*) in tomato. The elicitor acibenzolar-S-methyl (ASM) was also effective against *P. infestans* on tomato (Becktell *et al*., 2005). In van der Wolfe *et al.,* (2012), seed treatments of BABA did not significantly reduce *Phytophthora parasitica* on cabbage (*Brassica oleracea*), however, there was a greater degree of pathogen reduction when BABA was applied to the leaves. In a study by Ji *et al*., (2011), ASM was found to supress *Phytophthora capsici* on squash by an average 75%, and by about 80%. Elicitors are best used preventatively and do not give 100% control (Sillero *et al*., 2012; Walters *et al.*, 2013), but can help in reducing the requirement for synthetic pesticide usage. As with varietal resistance, there can be fitness costs to plants associated with the defence response perhaps resulting in reduced yield, and the secondary metabolites produced have potential to negatively affect the quality or taste of crops.

The efficacy of elicitor agents varies with host genotype. A study by Sharma *et al.,* (2010) found that tomato genotypes varied significantly in their expression of BABA-induced resistance to *Phytophthora infestans*. In this study, the level of induction was not always related to the resistance rating of the tomato accession, and was significantly influenced by the pathogen isolate. The degree of resistance induction tended to decrease with increasing leaf age, possibly reflecting the effect of BABA from root to shoot. Sharma *et al.,* (2010) also found that, though BABA performed well on one isolate, it was less effective on two or three mixed isolates. In this case, it was suggested that studies focusing on one isolate or a single host genotype might lead to a misleading conclusion concerning the effectiveness of the elicitors in practice (Walters *et al.,* 2013). There is large body of work on the elicitor beta-amino-butyric acid (BABA), but toxicological as well as biodegradation studies are required before registration (Cohen, 2002).

Tables 12a to 12g have been created following the literature search for work on elicitors on each of the pathogens given in Tables 4 & 5 above. They show a range of products reported as reducing the severity of foliar infection by oomycetes on tomato, potatoes, leafy salads and ornamentals. Published research on elicitors used on ornamental plants and trees is very sparse (other than for phosphite whose fungicidal activity means elicitor activity is masked). It should be noted that in many cases there was significant disease reduction, but that this still resulted in a noticeable disease severity. However, in inoculated studies the conditions are usually made optimum for the pathogen and the plant receives a large amount of spores all at once. When infection occurs naturally there can be a more gradual challenge to the host and it is more likely that the plant would have more time to build up a defence.

Table 12a. Elicitor efficacy experiments carried out on Tomato blight caused by *Phytophthora infestans*

Product & main ingredients	Application method & dose	Crop growth stage	Conditions	Level of *P. infestans* control	Reference source
BABA	1g /L BABA to near runoff	30 day old (5-6 compound leaves)	Glasshouse 23 °C day, 18 °C night	Induction differed between cultivars and inoculum used, but in general there was less reduction in lesion size with increasing leaf age. Reductions in area under the disease progress curve compared to the water control; 43-100% on youngest leaf, 15-100% on oldest.	Sharma *et al,* 2010
Biofertilisers: Alfalfa extract / protein hydrolyzates from *Medicago sativa* Biosol PEN Autoclaved filtrate from a suspension of dried mycelium of *Penicillium chrysogenum* BioFeed QUALITY (AgroBio, Wageningen, NL). *Ascophyllum nodosum* and *Fucus* sp. extract with humic and fulvic acids Control: BABA (Sigma-Aldrich, Germany)	4% v/v Alfalfa extract, 0.1% v/v PEN and 2.5% v/v QUALITY five times weekly watering with 50 ml from 7 days after transplanting 10 day old seedlings until 1 day before inoculation 1g/L BABA sprayed 1 week before inoculation until run-off	7 to eight week old	Glasshouse 22 °C day, 18 °C night	Reductions in area under the disease progress curve shown compared to the water control: Alfalfa extract 23–78%, PEN 21–77%, QUALITY 17–66%, BABA 37–100%	Sharma *et al*, 2012
Vermicompost	Vermicompost with water at a 1 : 2 ratio	Unknown	Field Conditions with av. temp of 10.2 °C	Half as many plants infected than water control, but with a similar severity of infection	Zaller 2006

Table 12b. Elicitor efficacy experiments on Late blight of potato caused by *Phytophthora infestans*

Product & main ingredients	Application method & dose	Crop growth stage	Conditions	Level of *Phytophthora infestans* control	Reference source
Pseudomonas. fluorescens	2.5 g/kg. Bio agents were mixed with sand and distributed into the furrow at planting.	Applied at time of sowing	Field conditions	68.7% (compared with water control)	Basu *et al* 2001
Bacillus subtilis	2.5 g/kg Bio agents were mixed with sand and distributed into the furrow at planting.			55.5% (compared with water control)	Basu *et al* 2001
Gliocladium virens	5.0 g/kg Bio agents were mixed with sand and distributed into the furrow at planting.			61.5% (compared with water control)	Basu *et al* 2001
Trichoderma	1×10^6 spore/ml		Field in China with frequent rains & warm temperatures	Trichoderma strain HNA14 was comparable to Thiram fungicide	Yao *et al*., 2016
NOCC (dissolved chitosan)	0.1% (w : v) spray		Field conditions	NOCC showed greater inhibition than Ridomil fungicide	O'Herlihy, Duffy & Cassells, 2003
COS-OGA (cationic chitosan oligomers)	0.5% (v/v)	Five leaflet stage	Greenhouse conditions 16 h photoperiod. 20°C /16 °C day/night	Significant decrease in disease progress with 50% protection conferred	Clinckemaillie *et al.,* 2017
Trichoderma atroviride	Experiment 1 Tested *T. atroviride* against Bravo 500F fungicide Experiment 2 *T. atroviride* spores at (3×10), (3×10^2), (3×10^3), (3×10^4) & (3×10^5) Experiment 3	At planting for Experiment 1. Experiment 2 and 3 were carried out *in vitro*.	Plants were grown in a growth chamber for 4 weeks using artificial lighting.	Experiment 1 *T. atroviride* alone and in combination with Bravo 500F reduced disease severity by 27% and 36% Experiment 2 Blight severity reduced with increasing *T. atroviride* cfu: 3×10^5 (0%), 3×10^4 (21%), 3×10^3 (21%), 3×10^2 (34%) & 3×10 cfu/mL (40%) Experiment 3	Al-Mughrabi,K., 2008

Product & main ingredients	Application method & dose	Crop growth stage	Conditions	Level of *Phytophthora infestans* control	Reference source
	With blight mating types A1 and A2 *T. atroviride* spores at (3 × 10), (3 × 10^2) & (3 × 10^3) Trichoderma atroviride (3 × 10^4) Trichoderma atroviride (3 × 10^5)			The highest inhibition of the A1 mating type was obtained when the media was amended with 3 × 10^5 CFU/mL of *T. atroviride* (91.8%) followed by 3 × 104 CFU/mL (91.69%), 3 × 103 CFU/mL (90.94%), 3 × 102 CFU/mL (90.24%) and 3 × 10 CFU/mL (89.9%)	

Table 12c. Elicitor efficacy experiments on Basil downy mildew caused by *Peronospora belbahrii*

Product & main ingredients	Application method & dose	Crop growth stage	Conditions	Level of *P. belbahrii* control	Reference source
acibenzolar-S-methyl (ASM, Actigard 50WG),	Three applications at weekly intervals pre-inoculation and again post-inoculation either 3 or 7 days after inoculation. Foliar spray applications until runoff. Nine experiments with a range of rates between 30 – 500 mg l^{-1}. Drenches of 15ml into the 10cm pots were only tested with ASM.	From pre-inoculation 12-20 days after sowing cv Genovese in pots until 7 weeks later.	Glasshouse set to 23.8 °C, but it fluctuated depending on the ambient solar radiation. Relative humidity kept high	Significantly fewer sporangia than on non-treated control. ASM ranging from 25 to 400 mg/ L sig. reduced disease severity compared with non-treated. Sprays of 50 mg/L starting 3 days post-inoculation gave a 93.8% reduction in disease severity, whereas if left 7 days reduction was 47.1%. Pre- plus post-inoculation treatment gave the best results. Drenching sometimes reduced disease compared with the untreated, but was less effective than foliar.	Mersha *et al*, 2012
DL-3-aminobutyric acid (BABA),				Six, weekly, foliar sprays of BABA at rates equal or higher than 125 mg/L significantly suppressed downy mildew compared with the non-treated control.	
isonicotinic acid (INA),				INA gave inconsistent, low level disease reduction	
salicylic acid (SA				SA gave inconsistent, low level disease reduction	
sodium salicylate (SS)				SS gave inconsistent, low level disease reduction	

Product & main ingredients	Application method & dose	Crop growth stage	Conditions	Level of *P. belbahrii* control	Reference source
acibenzolar-S-methyl (Actigard)	50 mg/L sprayed on all six growth stages one week after the last seeding. A week after ASM treatment, plants were inoculated with *P. belbahrii*	Four cv. Genovese in pots at 2,3,4,5,6 & 7 weeks old (9 pots per age)	Glasshouse set to 23 C°; however, temperature varied ± 5 °C depending on the ambient solar radiation.	One application of ASM before inoculation significantly reduced disease severity except for 2-week-old basil (when plants are very susceptible to the disease). N.B. a significant difference still left e.g. 47% mildew compared with 56% in the water control. The efficacy of ASM treatments tended to increase with the older plants were at inoculation. ASM was stated to be best applied to 5 to 7 week old plants before infection.	Patel *et al*, 2014
Six products tested:	Foliar spray by hand-held sprayer in 800 L ha water. Applied twice at a 6 day interval. Inoculated by misting spray of spores 24 hours after last treatment.	70–80 cv. Genovese Basil seedlings / 1.5 L pot. Treated 16-21 days after sowing, at third true leaf stage.	Glasshouse 85 –95% humidity,19°C - 25°C	For most products, significant reduction in incidence (proportion of basil plants with downy mildew) & severity compared with untreated. At 20 days, after last treatment, Trial 2 elicitor results similar to azoxystrobin fungicide. Results for untreated control plants to compare with treated plants Trial 1. 89% incidence / 67% severity, Trial 2: 84% incidence / 62% severity	Gilardi *et al.* , 2012
Bion (acibenzolar-S-methyl)	1 g a.i. /100 L			Trial 1: 49% incidence / 33% severity Trial 2: 8% incidence / 5% severity	
Regalis (prohexadione-Ca)	5 g a.i. /100 L			Trial 1: 60% incidence / 42% severity Trial 2: 25% incidence / 18% severity	
Glucoinductor (glucohumate activator complex)	400 g a.i. /100 L			Trial 1: 31% incidence / 22% severity Trial 2: 4% incidence / 2% severity	
Alexin (Phosphite fertiliser P_2P_5 52% K_2O 42%)	130 + 105 g a.i. /100 L			Trial 1:12% incidence / 9% severity Trial 2: 2% incidence / 2% severity	

Product & main ingredients	Application method & dose	Crop growth stage	Conditions	Level of *P. belbahrii* control	Reference source
Ortavia (azoxystrobin)	18.6 g a.i. /100 L			Trial 1: 18% incidence / 12% severity Trial 2: 1% incidence / 0.6% severity	
Thyme oil extract	100 g a.i. /100 L			No significant differences from Untreated	

Table 12d. Elicitor efficacy experiments on Lettuce downy mildew caused by *Bremia lactucae*

Product & main ingredients	Application method & dose	Crop growth stage	Conditions	Level of *B. lactucae* control	Reference source
Phytogard	5.8 ppm foliar spray	14 day old seedlings	Growth Cabinet at day 20°C ± 0.1, night 16°C ± 0.1	Disease Index of 4.6 and an efficacy of 41% at 5.8 ppm	Pajot, Le Corre & Silue, 2001
	29 ppm foliar spray			83% efficacy at 29 ppm	
	40.6 ppm, 58 ppm & 87 ppm foliar sprays			Complete protection at 40.6 ppm and above	
BABA	10 mM foliar spray			Disease Index lower than 0.2 at 10mM	
	20 mM, 30mM, 50 mM & 80 mM & 100 mM foliar sprays			Complete protection, with zero phytotoxicity, at 20mM & above	

Table 12e. Elicitor efficacy experiments on Onion downy mildew caused by *Peronospora destructor*

Product & main ingredients	Dose	Crop growth stage	Conditions	Level of *P. destructor* control	Reference source
Fungal elicitor	0.03%	Not stated	Field	Highly effective	Dmitriev & Grodzinsky, 1987

Table 12f. Elicitor efficacy experiments on Hebe downy mildew caused by *Peronospora grisea*

Product & main ingredients	Dose	Crop growth stage	Conditions	Level of *P. grisea* control	Reference source
Bion WG50 (acibenzolar-S-methyl)	0.1g/L	8-10 leaf stage	Transparent airtight containers 20°C day 18°C night	73%	Stapel & Guerrand, 2010 (Efficacy Test)
PrevB2. Extract of citrus fruit peelings (terpenes) enriched with boron	10ml/L			Not reported	
Semafort. Mixture seaweed extracts, amino acids & fertilizing elements NPK	7.5ml/L			85%	
Bion WG50 (acibenzolar-S-methyl)	0.1g/L	Weeks 25 - 49	Greenhouse & tunnel	0, -10% & 35%	
PrevB2. Extract of citrus fruit peelings (terpenes) enriched with boron	10ml/L			90%, 92% & 92%	
Semafort. Mixture seaweed extracts, amino acids & fertilizing elements NPK	7.5ml/L			13%, 19% & 14%	

Table 12g. Elicitor efficacy experiments on Rose downy mildew caused by *Peronospora sparsa*

Product & main ingredients	Dose	Crop growth stage	Conditions	Level of *P. sparsa* control	Reference source
Biochikol 020 PC (chitosan)	0.025 %	Shrub	Tunnel	Over 72%, similar to standard fungicide controls. Increased dosages of Biochikol 020 PC found a reduction in efficacy.	Wojdyła A.T., 2004

Discussion

A large amount of work has gone into the development of elicitors, however there is still a significant amount of work that needs to be done. Further work is required to develop the use of elicitors in the field as shown by Storer *et al*., (2016). Microbial elicitors could perform differently when being used in a commercial setting due to the varying environment as opposed to being used in the lab. Walters*.,* (2013) has found that plants grown outside typically have already been induced or primed from their interactions with the biotic and abiotic stresses found. This may make the application of elicitors in the field less feasible, due to the decrease in eliciting activity of the compounds when compared to the laboratory assays.

Increased independent research is required to provide both growers and agronomists with knowledge on how best to utilise them, as some elicitors are not designed to be used in a good year but rather a bad year and bring the yield up to an average year (Storer *et al*., 2016).

The development of DRASTIC gene expression database can aid both the agronomists, in selecting the correct elicitor for the issue and researchers to search for potential new candidates for efficacy work. There is still a large gap in knowledge on how induced resistance can be used in an integrated cropping system, there are many factors which can affect how effective an elicitor can be and it can often be host species specific. All of the information provided by drastic is based on work done on the model plant Arabidopsis, making it hard to transfer to a commercial crop.

The elicitor effects are wide ranging depending on the substance used, and can provide a significant benefit to a grower in control of pathogens as seen in Tables 11 and 12. Some of the results vary, however this could be due to the host variability. As seen with the effect of Actigard in Patel's (2014) study, it varies based on the age of the basil plant that is treated, the older the plant the better the control. However other work has stated that earlier application of an elicitor at the propagation stage before the growers receive the plants could provide a benefit against pathogens (Holden 2016). This would allow it to fit into an integrated management plan, lowering the incidence of disease in the field and reducing the amount of fungicides.

Elicitors rarely provide 100% control of the issues as seen in Tables 11 and 12, this limitation combined with the variable environment in which plants are grown can make it hard to successfully implement elicitors into crop production. Extensive testing will have to be carried out on a crop by crop basis, taking into account the cropping system used, the pest/pathogen problem and the plant pathogen interaction (Burketova *et al*., 2015).

Another issue raised against elicitors is the fitness and allocation cost of using them. When elicitors are used it requires the plant to redirect metabolites and energy from the growth and development of the plants, this can result in the stunting of the plant and the potential for yield loss in some crops. There is also the potential ecological cost that results when one of the expressed defence traits has a negative effect with the environment such as mycorrhizal associations which are common in soil grown crops (Heil and Baldwin, 2002). Work on wheat has found that the application of elicitors can negatively impact the crop when they have been applied and no pathogens were present, this shows that there is a potential allocation cost. This is even more evident when the plants were under nitrogen deficiency (Csinos *et al*., 2001; Latunde-Dada and Lucas, 2001; Ziadi *et al*., 2001; Prats *et al*., 2002). However the use of elicitors on beans by Iriti and Faoro, (2003) found that there was no significant fitness cost to the plant and found that there may even be a yield increase when no disease pressure is present. This suggests that some plants store more resources than are needed and can allocate these where needed. Biostimulants work best when the plant is under stress, with a higher effect the more the plant is stressed (Pers. comm. Frederik Van Baelen, 20 Dec. 2019).

While jasmonic acid is vital for regulating the resistance to pest attack salicylic acid is responsible for regulating defences against pathogens. There are numerous reports of one negatively influencing the other, the upregulation of jasmonic acid downregulates the salicylic acid, and the other way around. This can make the plants more susceptible to pest attacks if an elicitor for pathogens is applied (Bostock, 2005; Stout *et al*., 1999; Thaler *et al*., 1999, 2002; Nike *et al*., 1998; Glazebrook *et al*., 2003). Salicylic acid is thought to induce resistance against biotrophic pathogens and some pests that feed on the phloem. Induction of salicylic acid by *P.syringea* has been found to increase *Arabidopsis* plants susceptibility to a necrotrophic pathogen *Alternaria brassicicola* by supressing the jasmonic acid pathway (Spoel *et al*., 2007). This suggests that the infection from one type of pathogen lifestyle will compromise the defence against the other and that other external regulatory mechanisms control plant defences.

There is a confusion in the use of elicitors, many substances such as Phosphite and seaweed extracts are found in popular foliar fertilisers such as Hortiphyte and Maxicrop. These are commonly used and there is no limit as to how much can be applied, however Phosphite is known for its fungicidal effect with a few registered fungicide products such as Frutigard (MAPP No 19105) and Soriale (MAPP No 19166). Phosphite as a fertiliser has been found to be a poor source of phosphorous, this is due to the fact that phosphite is a stable molecule and harder to breakdown within the plant meaning it persists in an unusable state for long periods of time (Gómez-Merino and Trejo-Téllez, 2015).

Seaweed extracts are reported to have activity against oomycete (Montenegro *et al*., 2019) this is in conjunction with helping nutrient uptake. Seaweed extract is a product that has a duel function, it can increase nutrient uptake and also aid in pathogen control. This makes it hard to identify what category it fits into, according to new EU laws this is left to the local government. There are a wide range of methods to extract the benefical chemical compounds, each company that produces seaweed-based products uses a different proprietary method. These methods vary and include alkali extraction, acid extraction and cell bust technology (Battacharyya *et al*., 2015). The composition of the extract can vary based on the extraction methods, as such the activity of the same raw seaweed material can be different depending on the extraction process used (Kim, 2012, Khairy and El-Shafay, 2013). Chemical compounds found in seaweed extracts are known to help improve the growth and root colonization of beneficial soil fungi and is known to help improve the hyphal growth of Arbuscular mycorrhizal fungi (Kuwada *et al*., 2000; 2006).

June 2019 saw the EU approve new regulations for fertilisers which will come into effect in 2022. This gives a clear definition of what is considered a biostimulant and will be completely separate from plant protection products. Plant biostimulants will now only be considered a fertiliser if the product stimulates plant nutrition processes independent of the products nutrient content with the sole aim of improving:

- Nutrient use efficacy
- Tolerance to abiotic stress
- Quality traits
- Availability of confined nutrients in soil or rhizosphere

These are divided into microbial and non-microbial plant biostimulants. This however doesn't cover the products that elicit activity against plant pathogens. And where two effects (such as nutrient use efficacy and eliciting plant defence mechanisms) are had from one product, it is not covered by the EU regulations and is only covered under each individual state's laws (EU 2019).

Conclusions

While there has been a significant amount of research done on elicitors, more is still required to ascertain its effectiveness in the field and any economic effects it might have. The interactions between the elicitors, the plant and the pathogen have to be further explored, as host specificity can have a significant impact on how effective an elicitor can be.

Furthermore timing of application needs to be developed to maximise each application. This should be combined with how effective it is to prime the cells rather than to induce resistance in the plant.

The impact that natural elicitors from various stresses have on a crop will affect how well additional applications of elicitors will perform (Walters *et. al* 2013), work is required to ascertain whether or not elicitors provide a significant economic benefit when used on outdoor soil grown crops, or if they should only be used for crops grown under protection (more isolated from natural elicitors) to maximise their benefit.

More research needs to go into the effect of elicitors when they are resource deficient and whether or not there is a fitness cost to the application of elicitors. Direct induction of defences may not be necessary and has potential to cause more harm than good, priming of the cells however has the potential to have less of a fitness cost associated with it. However Walters *et al*., (2009) found that it may only show a benefit when there is a higher disease pressure. As discussed by Spoel *et al*., (2007) the application of an elicitor that upregulates the salicylic acid potentially inhibits the jasmonic acid pathway leaving it susceptible to necrotrophic pathogens such as *Botrytis cinerea*. Research must be carried out on this aspect so as to guide growers on the appropriate action, as applying an elicitor that upregulates the salicylic acid when it is not needed could result in crop lose due to an infection from a necrotrophic pathogen.

Seaweed extracts benefit the plants in various ways, these benefits are small but can easily be used to help improve overall plant health and can potentially help to improve resistance to pathogens. Their impact in the field will need to be assessed, but could be an invaluable tool against pathogens. Furthermore, the effect of the extraction processes on the activity of the resulting extract should be investigated and made clearer to growers, researchers and agronomists to better guide their efforts.

Phosphite has recently been registered in the EU as an active ingredient for plant protection having shown efficacy against oomycetes. It is still currently available as a component in many products that are sold as fertilisers or biostimulants, not as plant protection products.

Chitin is another product that shows great promise as an elicitor, it doesn’t help to improve nutrient uptake and as such will not be able to be included into the new EU fertiliser laws which covers biostimulants. AMF has potential to be very beneficial to outdoor production, its use in aiding against soilborne pathogens is well known, and its use against aerial oomycetes is not. Studies are required to find if AMF will have any effect on aerial oomycetes.

The elicitors mentioned above present some of the best options for control, due to the host specificity of some crops work will be required on each crop to ascertain the most suitable and economically feasible elicitor to apply.

Further Work Recommended

- A greater understanding of the plant defence mechanisms used by individual plant species (and how these differ between varieties showing enhanced resistance to a particular pathogen) to aid in elucidating the likely effectiveness of various elicitor products.
- More work on which type of chemical/non-biological or biological product has the ability to stimulate particular plant defence mechanisms.
- Further research is needed on the efficacy of products across a greater range of host variety/pathogen interactions.
- It will be important to determine dose rates and timings both in relation to growth stage and when to start and how often to repeat application.
- Laboratory / glasshouse experiments need to be followed up by trials within commercial crops.
- As evidence so far indicates incomplete control by elicitors, consideration should be given to disease levels that could be tolerated in individual crops following application (in a similar way to that of varieties showing levels of resistance).
- Wider evaluation of elicitors and the benefits or otherwise on pest and pathogen control, nutrient uptake and general plant health, particularly in field grown crops where there are usually more variables than under protection.
- The interaction between natural elicitors outside and their interaction with elicitors applied by growers.
- The effect of elicitors when used in resource deficient area.
- The fitness cost incurred by inducing the resistance when no resistance is required vs priming the cells at propagation.
- The interaction between the upregulation of the salicylic acid pathway and it impact on weaknesses incurred to necrotrophic pathogens.
- Many of these materials have yet to be approved for use in disease control programs – a notable exception to this is COS-OGA (Fytosave) which is approved for downy mildew management in protected/outdoor herbs, protected/outdoor lettuce, protected/outdoor brassicas, HNS and protected/outdoor roses.

Review/overview of Decision Support Systems/Tools (DSS/DST) for managing downy mildews

'Decision-support systems are tools that help growers to decide which [crop disease] management options to employ and to make (spray) decisions' (Wallhead & Zhu, 2017). To effectively deploy an integrated disease management program some form of decision support is essential. As outlined above, the levels of knowledge about specific downy mildew diseases vary greatly and so, not surprisingly, does the level and sophistication of DSS. DSS developed for downy mildews range in complexity from simple risk matrices (Jennings & Thorp 2016; Jennings *et al*., 2017) to models that contain sub routines predicting sporulation, inoculum concentration, infection, and latent periods in relation to meteorological data and using meteorological forecasts to predict disease risks. In the majority of cases the models have been developed as stand-alones and are perhaps better described as Decision Support Tools (DST). In the general area of downy mildew management in horticultural systems most advances have been made with *Plasmopara viticola* in grape vines where there is a substantial level of epidemiological modelling and understanding (Rossi *et al*., 2008; 2012a; Caffi *et al*., 2011; 2013) that has led to wide experience of field testing of DST (Caffi *et al*., 2010; Magarey *et al*., 2002) as well as the incorporation of downy mildew DST within full DSS designs and addressing the many issues that hinder/help the implementation of DSS, e.g. vite.net® and AusVit (Rossi *et al*., 2012b; 2014; Magarey *et al*., 2002). The more recent general DSS, vite.net®, is composed of two parts the first is concerned with real-time monitoring of vineyard parameters concerned with the air, soil, plants, pests, and diseases, whilst the second is the 'front end' – a web-based tool that uses models to analyse the data from this monitoring and provides the user with bespoke updates, alerts and advisory decision supports. Rossi *et al*. (2014 & 2019) felt that by addressing a number of key factors, most importantly

1) the holistic treatment crop issues (P&D, fertiliser use, agronomy and irrigation)

2) breaking complex decisions into straightforward 'decision supports

3) easy and rapid access *via* internet connection (no frustratingly-lengthy loading up procedures)

4) strong emphasis on the decision support and NOT decision-making role of DSS

5) two-way communication between users and providers to make it possible to consider and resolve site specific issues and information, the likelihood of potential users adopting the vite.net® DSS was greatly increased.

This process requires ongoing active support of the DSS/DST and is a far cry from the majority of downy mildew DST which are currently at an earlier phase of development and generally not presented to potential users as parts of holistic systems and often have quite difficult-to-handle 'front ends' as indicated by the preference of Australian lettuce growers in comparison trials for DOWNCAST (designed for onion DM) over the less straightforward BREMCAST (designed for lettuce DM) (Minchinton *et al*., 2010)

The downy mildews considered in this study all respond in relatively similar ways to environmental conditions (Table 1). Sporulation is induced by periods of high RH% (or leaf wetness) in darkness. As a result, sporulation/spores are often present on leaves at dawn following such a favourable night/early morning period and available for release. Spore release generally commences at sunrise with increasing solar radiation, increasing air temperature and reducing RH% at the leaf surface (Su *et al*., 2000) and in some cases, release is enhanced by vibration or air movement. Airborne conidia can be transported long distances, but the majority will settle out and land on plant surfaces nearby. Leaf surface wetness is needed for germination and infection to occur – usually a LWD of 2h or more is needed for germination and 3-5h for infection. Latent periods between infection and symptom appearance, where known, vary a little more but still generally fall between 5 and 10 days, except for *Peronospora destructor* in onions (9-16 days), which is recognised for its longer periods of latency. Greater variation is seen between the species in their response to temperature where it affects both the optima for processes like sporulation and infection as well as the lengths of latent periods. The species considered fall into two broad groups: cooler-loving species with optima for infection approximately between 10 and 15^{o}C and warmer-loving species with optima between 15-20^{o}C (Table 1).

Whether they are simple 'rules of thumb' like the '10:10:24' (**10**mm of rainfall when air temperatures are ≥**10**^{o}C and the soil surface remains wet for ≥**24**h) developed to determine conditions conducive to downy mildew of the vines (Magarey et al., 1993; Magarey, 2010) or relatively complex models the basic data inputs required remain fairly generic. All models require air temperature and the majority use relative humidity (RH%), the only exception being FSP for *P. sparsa* in boysenberry (Kim *et al*., 2014) which uses rainfall (Table 13). Rainfall measurements are used by several other DST, whilst the second most frequently used moisture parameter is plant (leaf) surface wetness (LW, Table 13). The majority of DST models utilise hourly/half hourly meteorological data and the quality and relevance of this data to the crops being managed is of key importance to the veracity of the warnings/advisories provided by the DST/DSS (Fall *et al*., 2016; Pfender *et al*., 2011).

With improvements in detection technologies allowing the rapid and precise identification and quantitation of pathogen propagules, there has been an increasing move towards direct monitoring of airborne inoculum to determine the disease risks (Choudhury *et al*., 2016; 2017; Klosterman *et al*., 2016; 2017; 2019; Kunjeti *et al*., 2016; Rahman et al., 2017; Wakeham *et al*., 2012; 2016). Indeed, without considering the level of inoculum or even the presence/absence of inoculum, growers are likely to spray unnecessarily if they follow meteorologically-based DST predictions literally. Another important consideration regarding the real-time *in situ* measurement of downy mildew inoculum in current UK horticultural crops is that, with the almost entire absence of reliable curative treatments in the fungicides available, there is a strong need to guide the timing of early applications of protectant treatments in relation to the presence and concentration of inoculum, to prevent early infection and disease establishment. The best disease risk forecasts are likely to result from simulations using a combination of meteorological and real-time inoculum measurements (Wakeham *et al*., 2016). A key problem with inoculum measurements is setting 'disease risk thresholds' as these will vary with inoculum potential or infection efficiency (IE) which is influenced by a range of factors such as crop variety/resistance, agronomic treatments such as fertiliser use, fungicide treatment history (usually accommodated within DST according to simple spray interval timing rules), plus other factors such as the crop plant microbiome composition/activity. Currently consideration of these factors is reliant on the local ('gut') experience and knowledge of individual growers and consultants. It is always important to remember that DSS/DST are risk predictors and should never be used in a prescriptive way but as Xu (2012) puts it; '[they should be used] more in the way of a weather forecast – when rain is forecast it is up to you – are you going to take an umbrella or coat with you? Or go out as normal?' The important question of IE feeds directly into the area of work in this project concerned with pathogen fungicide resistance/host disease resistance in *Bremia* populations in lettuce crops.

It is important not to lose track of the fact that as decision tools DST/DSS need not be overly intricate and that some very effective/useful assistance with crop management decisions can come from relatively simple systems. A good example of this is the risk tables drawn up from controlled environment observations for infection risks in sweet basil and aquilegia crops (Jennings *et al*., 2017; Jennings & Thorp, 2016), which, whilst not giving spray advisories, do provide a realistic estimate of when disease risks are low and the basis for the development effective protected environment management protocols. Also, it may be possible to adapt already-developed DST for other crops as seen in the Australian work with the DOWNCAST onion DST on lettuce and poppy crops (Scott *et al*., 2008; Minchinton *et al*., 2010).

Table 13. Summary of decision support tools and models that have been worked up for aerial oomycetes (predominantly downy mildews) considered in this study and the inputs the require to generate forecasts or aid decisions.

Pathogen	Crop	DST/DSS	Inputs
Bremia lactucae	Lettuce	BREMCAST (Kushalappa, 2001)	Hourly: Air temp., RH%, LW Disease presence +/- (scouting)
		California 1 (Wu *et al*., 2002 adaptation of Scherm *et al*., 1995)	Hourly: Air temp., RH%, LW, Solar Radiation *(model sets SR threshold of* $8Wm^{-2}$ *for start of 3h morning {'infection'} LWD)*
		California 2 (Kunjeti *et al*., 2016; Klosterman *et al*., 2016) Spore trapping qPCR – Risk thresholds	-
		Inagro/PCG/PSKW Glasshouse DST (van Hese, 2015) Basically, grower aims to keep RH% <90% - DST issues optimised spray advisories	Plant and harvest dates of crops Meteorological data is collected by 'climate box' installed in crop and sent to DST provider by internet
Hyaloperonspora parasitica	Matthiola/ Stocks	Rapeseed model (Neog *et al*., 2013) (very basic – not a DST as such)	Hourly: Air temp, Twice daily RH%, (daily rainfall)
Peronospora aquilegiicola	Aquilegia	Risk matrix (Jennings & Thorp, 2016)	Air temp., RH%
Peronospora belbahri	Basil	Risk matrix (Jennings *et al*., 2017)	Air temp., RH%
Peronospora destructor	Onions	MILIONCAST (Gilles *et al*., 2004)	Hourly: Air temp., RH%
		DOWNCAST modified (de Visser, 1998)	Hourly: Air temp., RH%, LW
		ONIMIL (Battilani *et al*., 1996)	Hourly: Air temp, RH%, Hourly/daily rainfall
		ZWIPERO (Friedrich *et al*., 2003)	Hourly: Air temp, RH%, LW, rainfall
Peronospora effusa	Spinach	Spore trapping qPCR – Risk thresholds (Choudhury *et al*., 2016; 2017) Possible development of recombinase-polymerase assays which will greatly improve this approach (Klosterman *et al*., 2017; 2019)	-
Peronospora sparsa	Rose	Aegerter *et al*. (2003)	Hourly: Air temp., LW
		Rose DST version 3.0 (Xu, 2012; Xu & Robinson, 2011)	≤ ½ hourly Air temp. RH%, LW, rainfall
		Fuzzy Peronospora Sparsa (FPS) Model (Kim *et al*., 2014)	Hourly Air temp., Daily rainfall
Phytophthora infestans	Tomatoes	BlightCast (www.syngenta.co.uk/blightcast) Used to access regional forecasts using Smith and Hutton criteria as a guide for extra vigilance with Greenhouse environmental controls (RH% and LW)	-
		EuroBlight – compares range of *P.infestans* DSTs for potato crops (Hansen et al., 2010 & ongoing)	Hourly: Air temp., RH%, rainfall

Fungicidal control in IPM programmes: work carried out at JHI

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.

M6.1 Protocols for fully validated laboratory tests to determine the fungicide sensitivity of isolates of Bremia lactucae, Peronospora farinosa f.sp. spinaceae, Peronospora viciae var. pisi (new target selected - Hyaloperonospora parasitica), Phytophthora infestans (tomato).

M6.2 Testing of up to 80 isolates (4 pathogens x 20 isolates) with 3 fungicides per crop and dissemination of results to industry and to inform WP3 O1 IPM programmes as appropriate (see M1.2)

A range of industry stakeholders and the AHDB crop protection team were consulted at the outset to select appropriate fungicides to be tested for each pathogen in this programme. Following discussion at the initial project meeting attended by stakeholders it became evident that there were few current options for the control of *P. viciae* and a decision was taken to replace this with testing of *H parasitica* on column stocks. Information was also drawn from the results of previously funded work on *Bremia lactucae*: (McPherson et al., 2011 PC 298; Wright, 2017 P07 and Mason & Jennings, 2019). The selected products are shown in Table 14.

Protocols (M6.1) were developed to test the selected fungicides and isolates of the pathogens obtained and tested for fungicide sensitivity (M6.2):

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. More than 100 sample packs and instructions (Figure 3) were posted to stakeholders along with postage-paid return. Growers were asked to sample four lesions per distinct outbreak wherever possible and to supply crop information. Additionally, an FTA card was supplied onto which four 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.

Table 14. Fungicides selected for testing on key crops

Crop	Product	Active Ingredient
Tomato		
Mandipropamid	Revus	250g/l mandipropamid
Cyazofamid	Ranman Top	160g/l cyazofamid
Fosetyl-aluminium	Previcur Energy	310g/l fosetyl-aluminium and 530g/l propamocarb hydrochloride
Column Stocks		
Mandipropamid	Revus	250g/l mandipropamid
Dimethomorph	Paraat	500g/kg dimethomorph
Metalaxyl-M	SL 567A	465g/l metalaxyl-M
Fosetyl-aluminium	Previcur Energy	310g/l fosetyl-aluminium and 530g/l propamocarb hydrochloride
Lettuce		
Azoxystrobin	Cleancrop Celeb	250g/l azoxystrobin
Dimethomorph	Paraat	500g/kg dimethomorph
Mandipropamid	Revus	250g/l mandipropamid
Spinach		
Azoxystrobin	Cleancrop Celeb	250g/l azoxystrobin
Dimethomorph	Paraat	500g/kg dimethomorph
Mandipropamid	Revus	250g/l mandipropamid

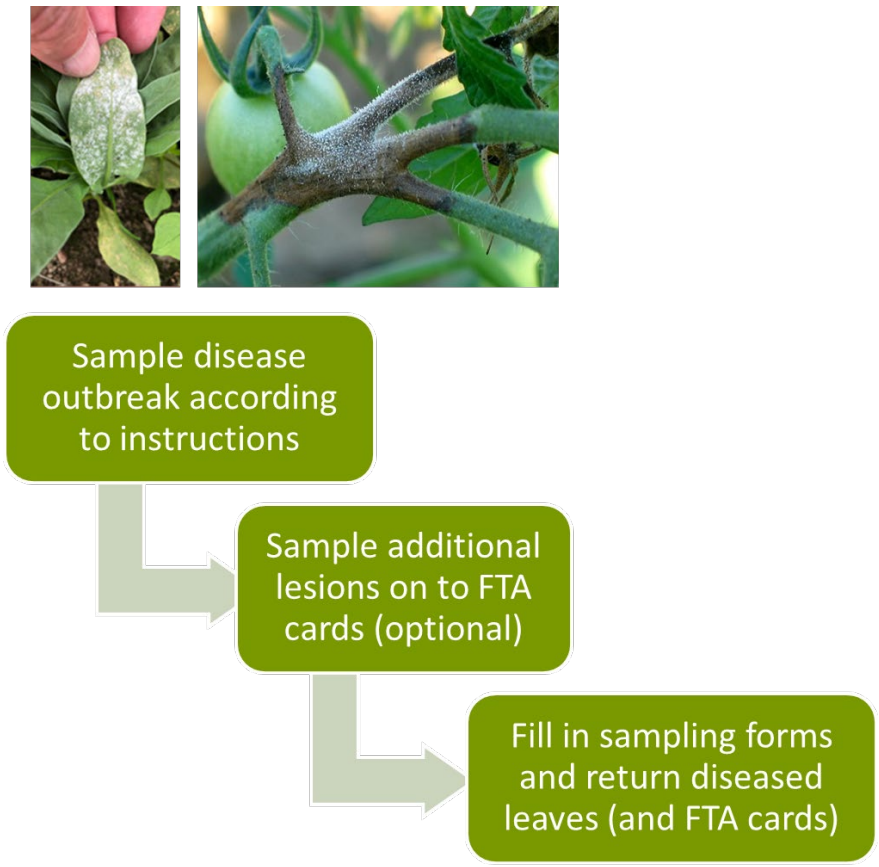

Figure 3. Sampling pack and instructions for oomycetes

Column Stocks:

In 2018 there was a serious issue with downy mildew in column stocks as reported by the National Cut Flower Centre, leading to some crops being unmarketable. There were anecdotal reports of resistance to some of the fungicides regularly used for downy mildew control. It was also apparent that a 7 to 10-day spray interval was not adequate. Subsequently, tests undertaken in 2018 on isolates from 5 nurseries (2 in Norfolk and 1 each in Lincolnshire, Northern Ireland and Cornwall) using 9 different fungicides showed that all isolates were totally resistant to metalaxyl M and that no chemical completely controlled the disease. The best control was obtained from Percos (ametoctradin and dimethomorph) and Paraat (dimethomorph) but one isolate also showed a low sensitivity to both of these products. Revus (mandipropamid), Fubol Gold (metalaxyl-M and mancozeb, but only from the Mancozeb) Fenomenal (fenamidone and fosetyl-aluminium) and Previcur Energy (fosetyl-aluminium and propamocarb HCl) gave moderate control whereas Ranman Top (cyazofamid) and Signum (pyraclostrobin and boscalid) gave almost no control.

The work carried out in 2019 builds upon these results:
Twelve isolates of *P. parasitica* originating from 6 outbreaks were isolated from diseased material supplied to the James Hutton Institute in 2019 (Table 3). Isolates were transferred to fresh, disease free leaves of *Matthiola indica* and were maintained for inoculum production by regular sub-culturing onto new plants.

Four fungicides selected by the industry (listed in Table 15) were tested at field rates only, to give a set of data that could be compared with similar work carried out in 2018. Plug plants were obtained from an industry stakeholder and grown on in an unheated glasshouse at the James Hutton Institute in July 2019. Fungicides were applied as a protectant treatment to whole plants (5 plants per concentration per isolate x 12 isolates). Inoculations were carried out 24 hours after fungicide application with a sporangial suspension of *P. parasitica* ($1x10^5$ spores/ml). Plants were incubated in darkness for 24h following inoculation and then under natural daylight conditions. Glasshouses were maintained at 15°C and 90% RH throughout incubation. Plants were assessed for disease incidence 14 days after inoculation by calculating the percentage of infected leaves per plant. Results were compared with the untreated control and percentage disease control was calculated as a proportion of the untreated control.

Table 15. Information relating to fungicides and application rates tested on *P. parasitica* isolates from column stocks.

Active ingredient	**Mandipropamid**	**Dimethomorph**	**Metalaxyl-M**	**Fosetyl-aluminium**
Product	Revus	Paraat	SL567A	Previcur Energy
Formulation	250g/l	500g/kg	465g/l	310g/l fosetyl-aluminium and 530g/l propamocarb hydrochloride
Max water volume (L)	1000	1000	10000	1000
Min water volume (L)	200	600	10000	200
Dose rate	0.6 L/Ha	3kg/Ha	1.3L/Ha	2.5L/Ha
Max tank mix (ppm)	750	2500	60.45	3875
Min tank mix (ppm)	150	1500	60.45	775

Table 16. Disease samples received and from which live isolates were obtained. Isolates with the same number and different letters originate from different samples within the same outbreak

ID	County	Sampled	Received	Live Sample obtained
2019_Pp1	Lincolnshire	19/05/19	20/05/19	Yes
2019_Pp2	Lincolnshire	19/05/19	20/05/19	Yes
2019_Pp3	Lincolnshire	19/05/19	20/05/19	Yes
2019_Pp4	Unknown	19/05/19	20/05/19	Yes
2019_Pp6A	Cornwall	26/06/19	04/07/19	No
2019_Pp6B	Cornwall	26/06/19	04/07/19	No
2019_Pp6C	Cornwall	26/06/19	04/07/19	No
2019_Pp6D	Cornwall	26/06/19	04/07/19	No
2019_Pp7A	Norfolk	03/07/19	12/07/19	No
2019_Pp8A	Lincolnshire	15/07/19	23/07/19	Yes
2019_Pp8B	Lincolnshire	15/07/19	23/07/19	Yes
2019_Pp8C	Lincolnshire	15/07/19	23/07/19	Yes
2019_Pp8D	Lincolnshire	15/07/19	23/07/19	Yes
2019_Pp9A	Norfolk	19/07/19	24/07/19	Yes
2019_Pp9B	Norfolk	19/07/19	24/07/19	Yes
2019_Pp9C	Norfolk	19/07/19	24/07/19	Yes
2019_Pp9D	Norfolk	19/07/19	24/07/19	Yes

Results (Column Stocks)

High levels of disease (90-100%) were observed in the untreated control plants, indicating that the test method and the inoculum quality of all the tested isolates was satisfactory (Figure 4). Average disease incidence varied between treatments (Figure 5)

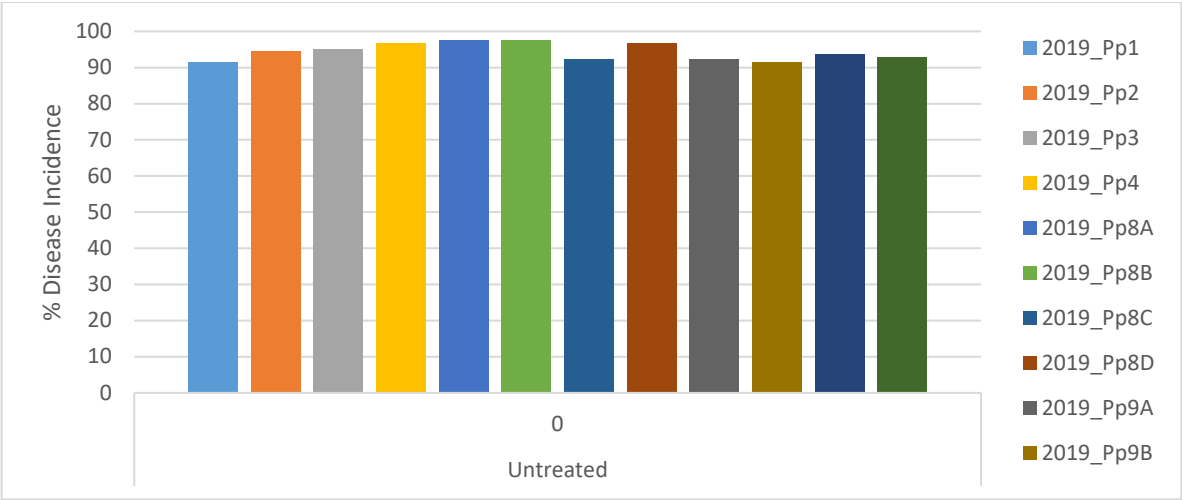

Figure 4. Average percentage disease incidence for each isolate of *P. parasitica* tested in the untreated control.

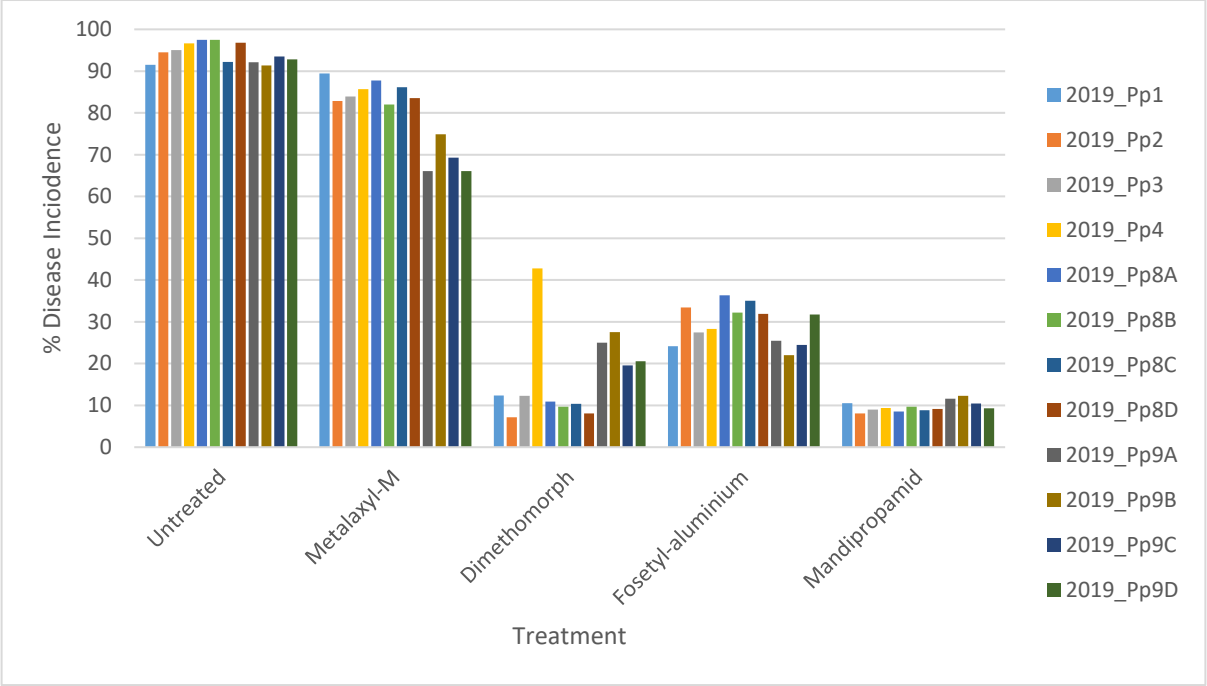

Figure 5. Average percentage disease incidence of downy mildew caused by isolates of *P. parasitica* on plants treated with a range of fungicide treatments applied at field rate.

The percentage reduction in downy mildew compared with the untreated control was calculated for each individual active ingredient tested. Mandipropamid (Revus) gave consistently high, but not complete, levels of disease control (80-86%) across all isolates tested (Figure 6), with no significant differences between isolates. Fosetyl-aluminium (and propamocarb hydrochloride) in the form of Previcur Energy gave variable levels of control (31-67%) across all isolates tested, with significant differences between isolates (Figure 7).

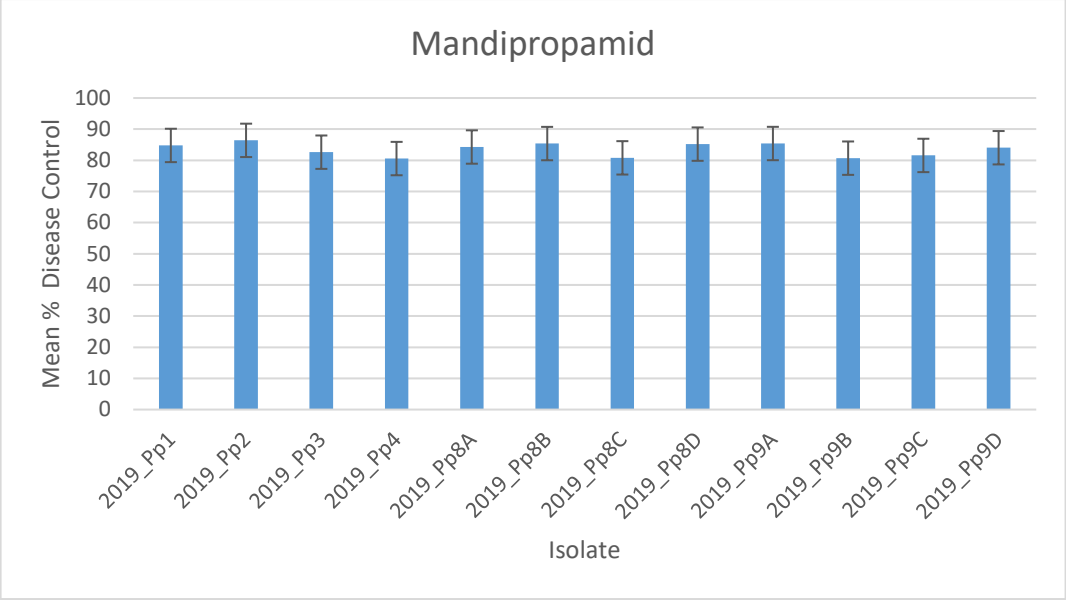

Figure 6. Mean percentage control by mandipropamid applied at field rate, of downy mildew on column stocks caused by 12 isolates of *P. parasitica*.

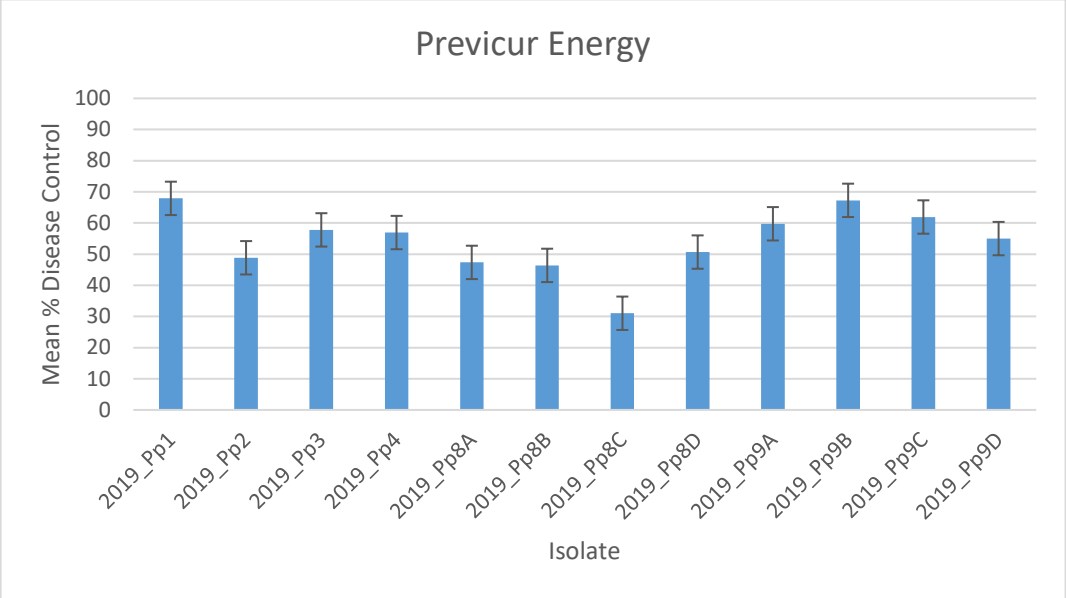

Figure 7. Mean percentage control by Previcur Energy applied at field rate, of downy mildew on column stocks caused by 12 isolates of *P. parasitica*.

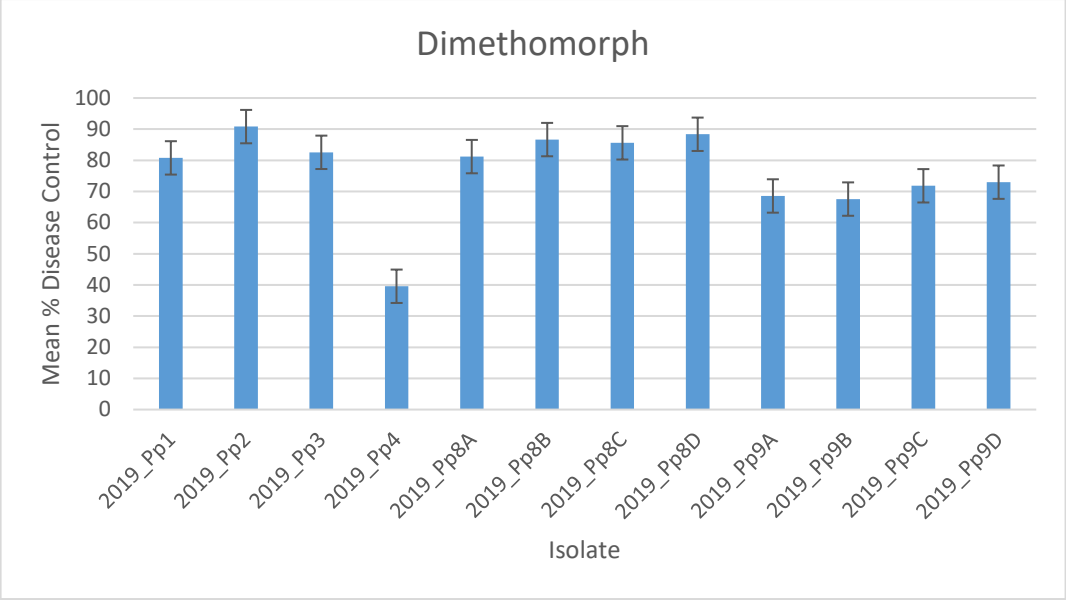

Figure 8. Mean percentage control by dimethomorph applied at field rate, of downy mildew on column stocks caused by 12 isolates of *P. parasitica*

Dimethomorph (Paraat) gave variable levels of disease control (Figure 8), with 80-90% control of all isolates apart from isolate Pp4 (40% control) and isolates Pp9A-D (68-72% control). Isolates Pp9A-D originated from one outbreak and therefore might be expected to clones; interestingly, an isolate tested from the same location in 2018 also showed reduced levels of control with dimethomorph despite little or no use of the product, suggesting carry-over of isolates between years (pers. comm). All isolates of *P. parasitica* were poorly controlled by Metalaxyl-M suggesting widespread resistance to the product (Figure 9).

The percentage disease control obtained by use of each product on average (mean of 12 isolates) is given in Figure 10. At the fungicide rates tested none of the products applied achieved 100% disease control. On average, mandipropamid (Revus) achieved 83% control, dimethomorph (Paraat), 76% control, Fosetyl Aluminium + propamocarb hydrochcloride (Previcur Energy) 54% and Metalaxyl -M 15% control.

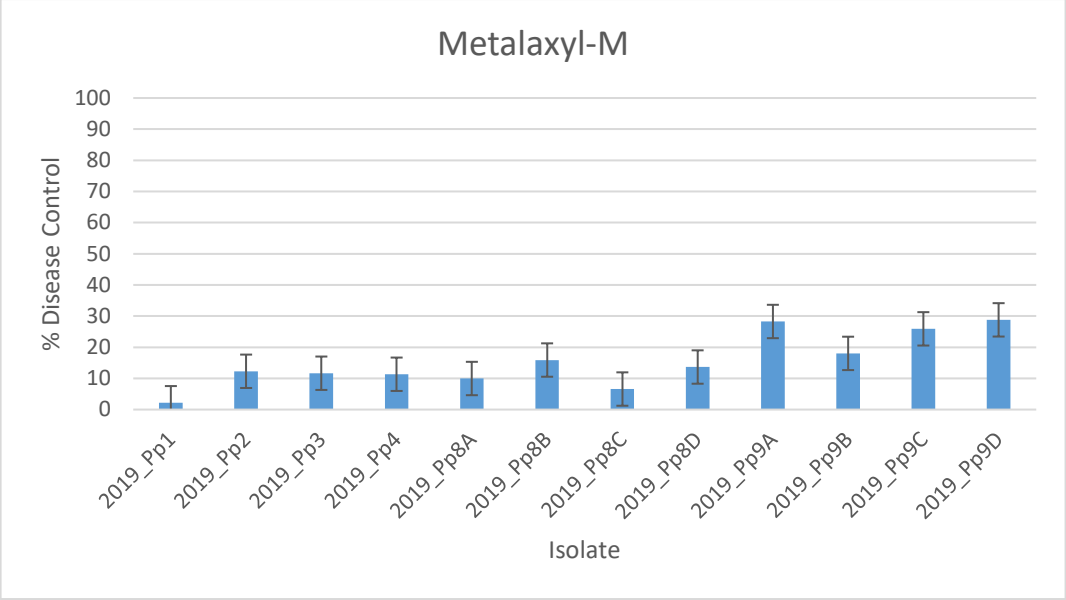

Figure 9. Mean percentage control by Metalaxyl-M applied at field rate, of downy mildew on column stocks caused by 12 isolates of *P. parasitica*

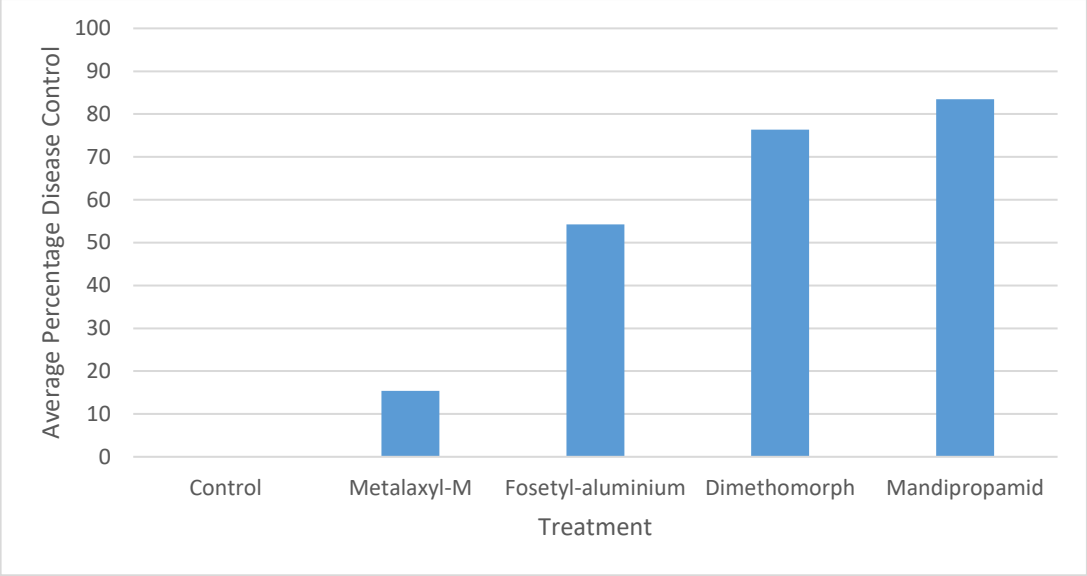

Figure 10. Average percentage disease control, compared with the untreated control, of all isolates of *P. parasitica* (n=12) treated with a range of fungicides**.**

Lettuce:

Samples were received from 14 individual disease outbreaks (BI1-14) in 2019**.** From these 14 outbreaks FTA samples were obtained from at least 1 lesion in 11 cases. Fifteen isolates from 10 individual outbreaks were successfully isolated from disease samples onto fresh leaf material and tested for fungicide sensitivity (Table 17).

Table 17. Isolates of *Bremia lactucae* tested for fungicide sensitivity in 2019. Isolates with the same number and different letters originate from different samples within the same outbreak

ID	County	Outbreak size	Date Sampled	Date Received
2019_BI10A	West Sussex	Scattered	06/08/2019	09/08/2019
2019_BI11A	Kent	Very Severe	15/08/2019	19/08/2019
2019_BI11B	Kent	Very Severe	15/08/2019	19/08/2019
2019_BI12A	Cambridgeshire	Patches	13/08/2019	19/08/2019
2019_BI12B	Cambridgeshire	Patches	13/08/2019	19/08/2019
2019_BI13A	Cambridgeshire	Patches	30/03/2019	04/10/2019
2019_BI1A	Fife	Scattered	29/05/2019	30/05/2019
2019_BI1B	Fife	Scattered	29/05/2019	30/05/2019
2019_BI2A	Dorset	Scattered	11/06/2019	13/06/2019
2019_BI2B	Dorset	Scattered	11/06/2019	13/06/2019
2019_BI3A	Leicestershire	Scattered	12/06/2019	14/06/2019
2019_BI5A	Cambridgeshire	Scattered	25/06/2019	27/06/2019
2019_BI7A	Surrey	Scattered	10/07/2019	12/07/2019
2019_BI7B	Surrey	Scattered	10/07/2019	12/07/2019
2019_BI8A	Norfolk	unknown	11/07/2019	18/07/2019

Fifteen isolates of *Bremia lactucae* were each tested for sensitivity to three fungicides at 4 concentrations as listed in Table 18. Fungicide concentrations were chosen based on existing information on use or baseline sensitivity testing of the individual products. Lettuce plants (cv. Green Towers) were grown from seed in potting compost. Two replicate pots each containing 2 plants were tested per fungicide per concentration. Fungicides were applied using a hand-held sprayer and plants incubated for 24 hours at 15°C before inoculation. Inoculation was carried out using a sporangial suspension ($1x10^4$ spores/ml) of *B. lactucae* obtained from diseased leaves and suspended in sterile distilled water according to the IBEB protocol. Inoculated plants were incubated under glasshouse conditions at 90%RH and 15°C for 10 days before disease assessment. Disease severity was scored across the two plants in each pot as percentage leaf area diseased. EC50 values were estimated by fitting a non-parametric spline to the disease score data obtained over a range of different fungicide concentrations. Interpolation was used to obtain the level of fungicide corresponding to the estimate of

disease severity at a point midway between the maximum and minimum disease severity values.

Table 18. Information relating to fungicides and application rates tested on *B. lactucae* isolates from lettuce.

Active ingredient	**Mandipropamid**	**Dimethomorph**	**Azoxystrobin**
Product	Revus	Paraat	Cleancrop Celeb
Formulation	250g/l	500g/kg	250g/l
Max water volume (L)	600	200	300
Min water volume (L)	200	200	150
Dose rate	0.6 L/Ha	0.36 kg/Ha	1.0L/Ha
Max tank mix (ppm)	750	900	1666
Min tank mix (ppm)	250	900	8333
Range of concentrations tested for EC50 calculation (ppm)	0, 10, 100, 750	0, 10, 100, 900	0, 100, 1000, 1666

Results (lettuce)

Moderately high levels of disease (33-65% of leaf area diseased) were observed in the untreated control plants, indicating that the test method and the inoculum quality of all the tested isolates was satisfactory (Figure 11). Dose responses for mandipropamid (Figure 12), dimethomorph (Figure 14) and azoxystrobin (Figure 16) were appropriate at the range of concentrations tested to calculate EC50 values. Data is also presented as percentage disease control of each isolate achieved following application of mandipropamid (Figure 13), dimethomorph (Figure 15) and azoxystrobin (Figure 17). In each case, the highest concentration of fungicide tested was representative of the maximum field rate and gives an indication of control under field conditions. The percentage reduction in lettuce downy mildew disease severity compared with the untreated control was calculated for each individual active ingredient tested. Mandipropamid (Revus) gave consistently high levels of disease control at field rate (91-100%) across all isolates tested (Figure 13). Similarly, dimethomorph (92-100%)

and azoxystrobin (81-97%) showed good control of lettuce downy mildew at the highest rates tested (Figure 15, Figure 17 respectively).

Estimated EC50 values (mean, minimum and maximum) for each fungicide are given in Table 19. This data provides a baseline from which to monitor any future changes in fungicide sensitivity. Some large differences in the min and max values were noted for some isolates, particularly for dimethomorph and azoxystrobin. No conclusions should be drawn from the data at this point as it may just represent variation and be irrelevant to in-field control. As the data set builds in future years this will bring clarity to these preliminary results.

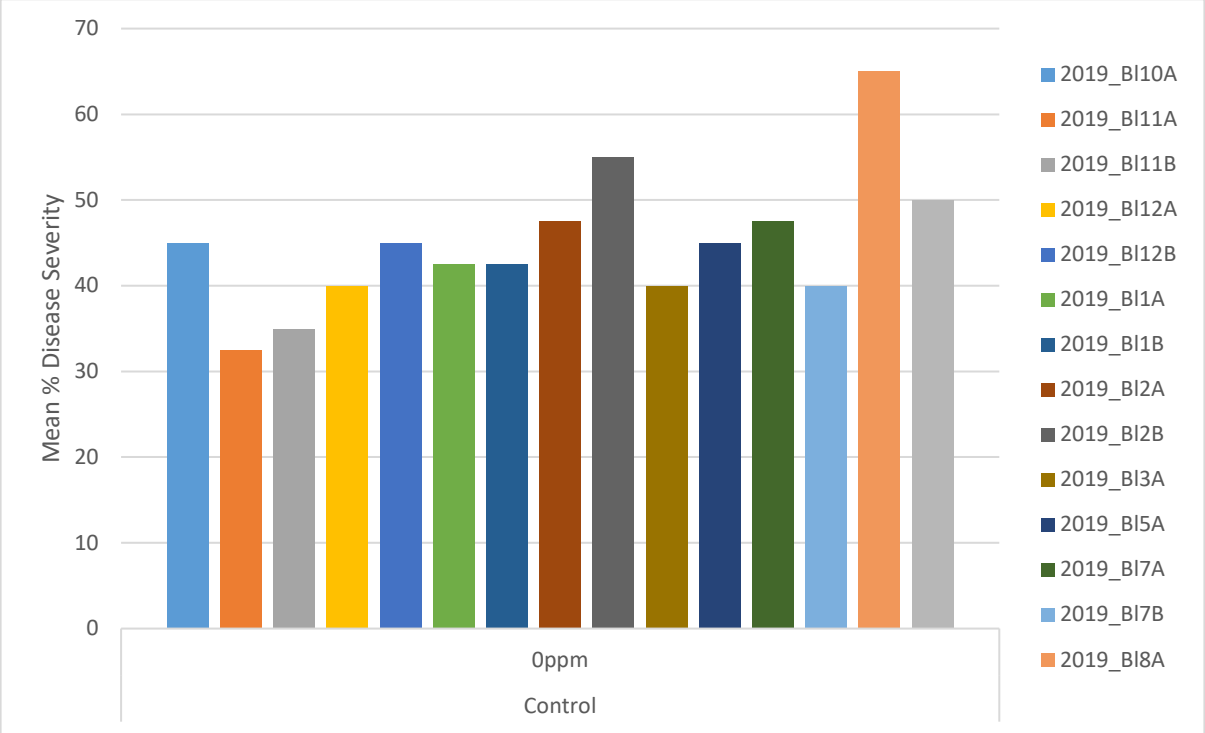

Figure 11. Average disease severity (percentage infected leaf area) for each isolate of *B. lactucae* tested in the untreated control.

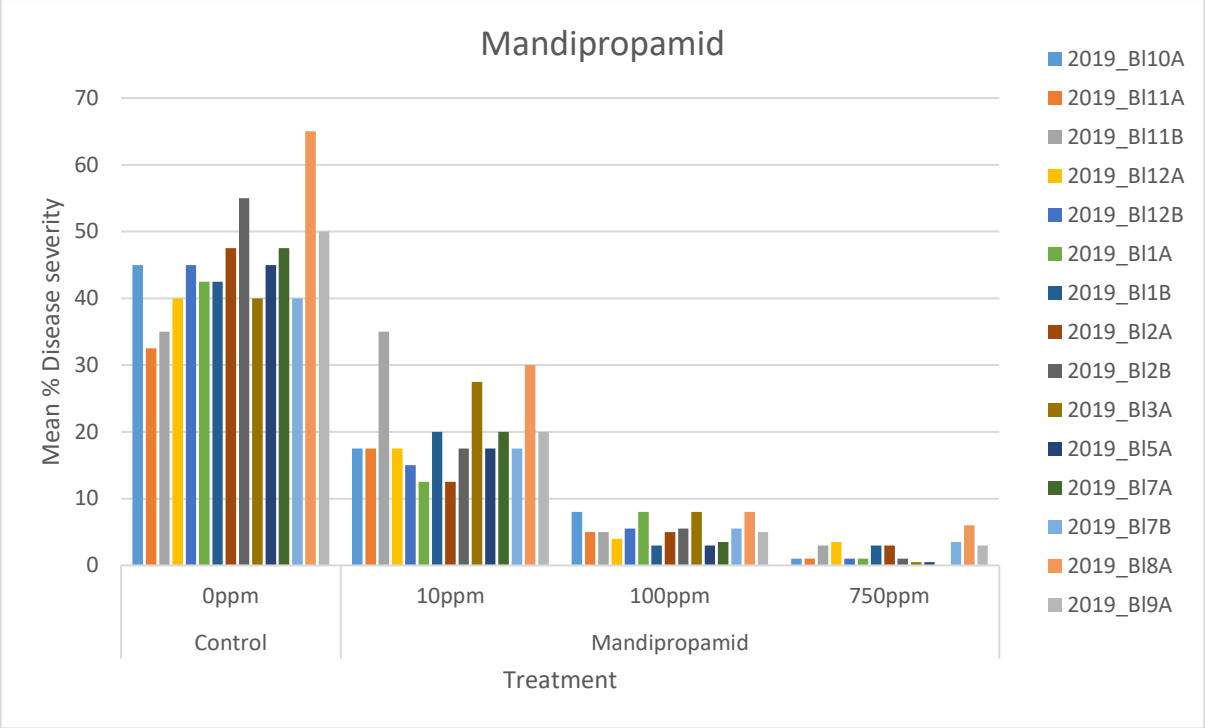

Figure 12. Mean disease severity (percentage of diseased leaf area) for 15 isolates of *B. lactucae* tested at 4 concentrations of mandipropamid.

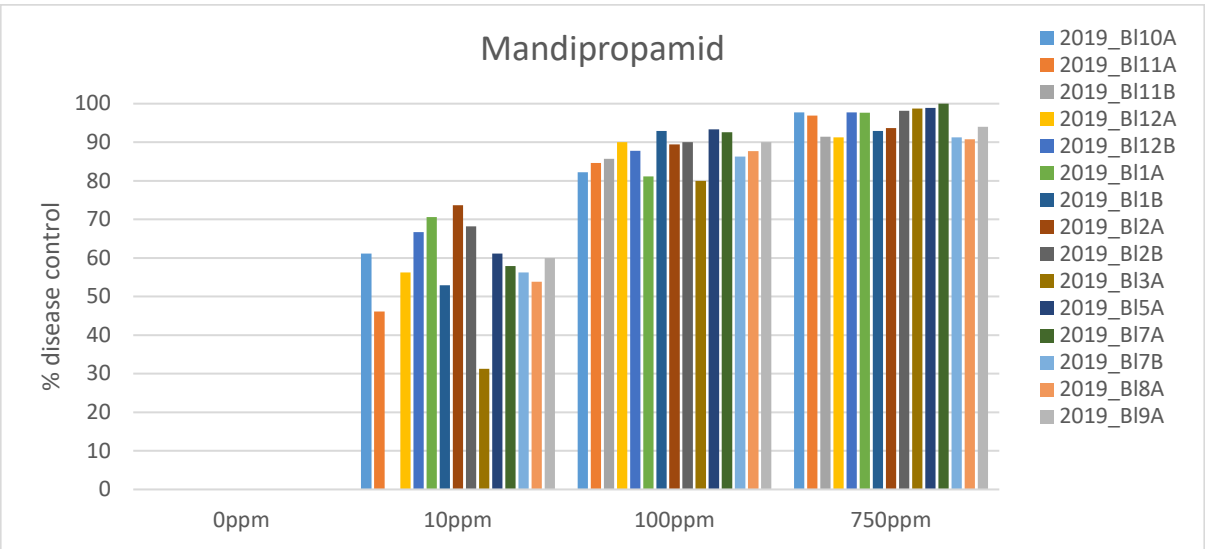

Figure 13. Mean percentage disease control of 15 isolates of *B. lactucae* by mandipropamid applied at a range of concentrations compared with an untreated control.

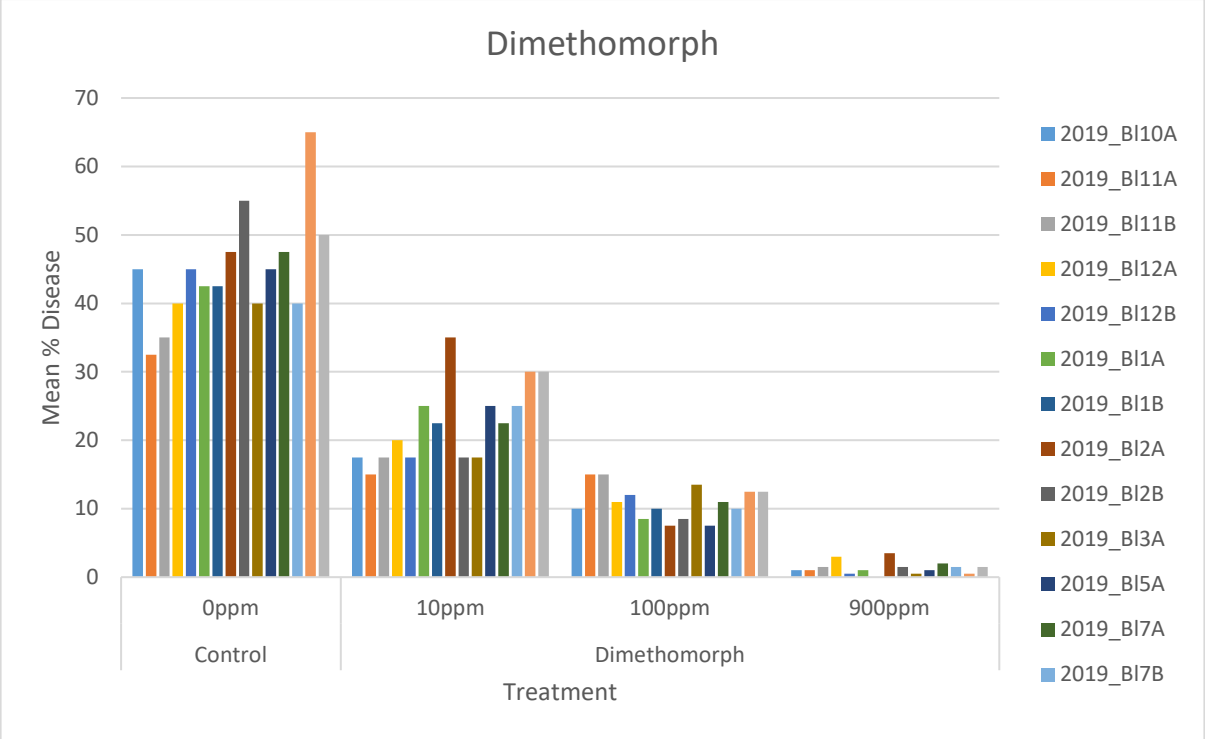

Figure 14. Mean disease severity (percentage of diseased leaf area) for 15 isolates of *B. lactucae* tested at 4 concentrations of dimethomorph.

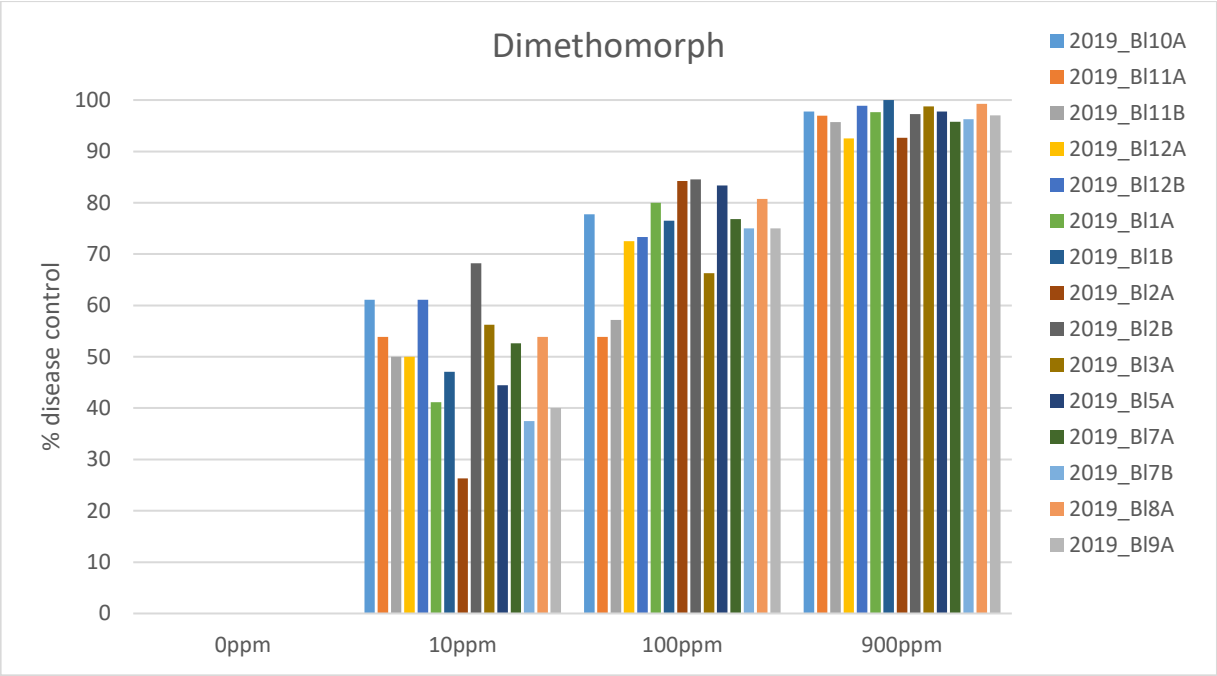

Figure 15. Mean percentage disease control of 15 isolates of *B. lactucae* by dimethomorph applied at a range of concentrations compared with an untreated control.

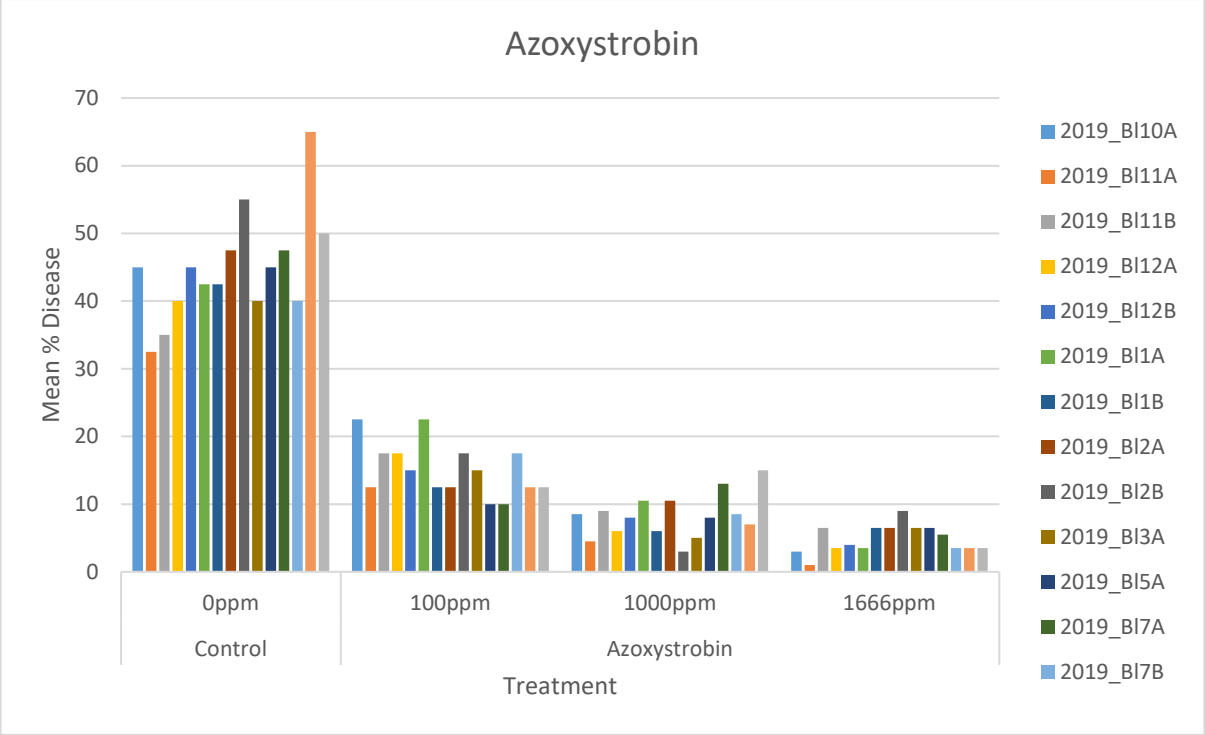

Figure 16. Mean disease severity (percentage of diseased leaf area) for 15 isolates of *B. lactucae* tested at 4 concentrations of azoxystrobin.

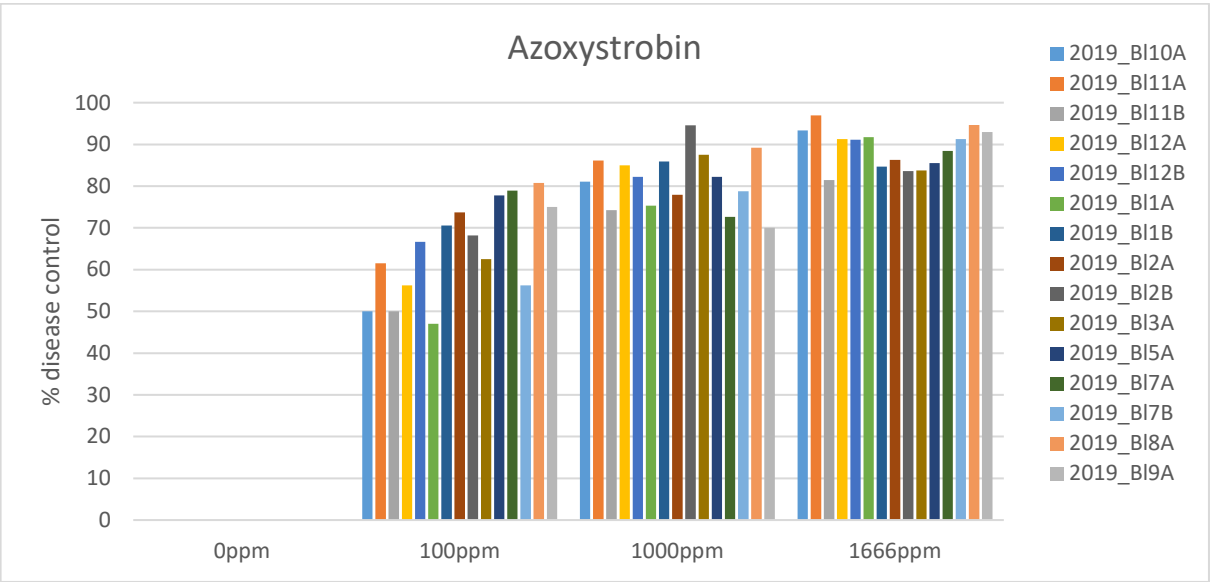

Figure 17. Mean percentage disease control of 15 isolates of *B. lactucae* by azoxystrobin applied at a range of concentrations compared with an untreated control.

Table 19. Mean, minimum and maximum EC50 values for mandipropamid, dimethomorph and azoxystrobin calculated from data obtained from 15 isolates of *B.lactucae* tested at a range of concentrations**.**

Fungicide	Mandipropamid	Dimethomorph	Azoxystrobin
Number of isolates	15	15	15
Mean EC_{50} (mg/L)	**15.6**	**38**	**233**
EC_{50} min (mg/L)	3.2	3.0	8.0
EC_{50} max (mg/L)	42.9	125	820

Tomato (*Phytophthora infestans*) and Spinach (*Peronospora effusa*)

No isolates of *P. infestans* were received from stakeholders in 2019 either within this project or through the AHDB Fight Against Blight campaign and fungicides tests could therefore not be carried out.

Isolates from 4 outbreaks in spinach were received late in the season (September/October). It became difficult under the prevailing light conditions to both grow robust plants and to sub-culture the isolates to produce sufficient high-quality inoculum for a fungicide test. Given concerns that the results would not be accurate it was decided to store the isolates until spring 2020, at which time we will attempt to revive the isolates from storage and carry out the tests under more optimal conditions.

Core Objective 7 - Lettuce Markers:

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.*

Backgound

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* 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 is 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 (e.g. 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.

M7.1 *Investigate the possibility of developing neutral and functional markers for Bremia lactucae populations. Obtain sequence information and collaborate with UC Davis to obtain and validate any existing markers. Obtain representative isolates of* Bremia lactucae *and set up DNA collections from the UK population and other international control isolates, differential sets of lettuce from IBEB with which to carry out race testing and identification*.

We have been in communication with Richard Michelmore and colleagues at UC Davis, USA and have received information and experimental details relating to marker development work in *B. lactucae* An AHDB-funded (CP 186) PhD student has been recruited to work alongside the project to develop these markers for use with GB pathogen populations in 2020. 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 additionally carried out preliminary race testing on isolates.

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. The test is illustrated in Figure 18.

Figure 18. Layout of differential set for race testing of one isolate of *B. lactucae*. Close up shows a positive reaction (susceptibility of differential to *B. lactucae* infection)

Results of *B. lactucae* race tests

Results of race testing of 15 isolates of *B. lactucae* are presented in Table 20. Eleven putative races were identified, of which one (2 isolates from outbreak Bl2) matched a race designated by the IBEB committee (Bl35EU). 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. It was therefore surprising that no isolates infected Dm3 (Dande) as this appears to be a commonly overcome race (from other available data).

Table 20. Results of race testing of 15 isolates of *B. lactucae*

	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		
2019_Bl3A	+	-	+	-	-	-	+	-	-	-	+	-	-	-	+	+	34-08-06	
2019_Bl8A	+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	50-10-02	
2019_Bl10A	+	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	50-10-02	
Bl:34EU																	54-15-01	
Bl:36EU																	55-15-01	
2019_Bl1A	+	-	+	-	+	+	+	-	+	+	+	(+)	-	-	+	+	58-30-06	
2019_Bl1B	+	-	+	-	+	+	+	-	+	+	+	+	-	-	+	+	58-30-06	
2019_Bl7A	+	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	62-03-06	
2019_Bl5A	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	62-15-00	
2019_Bl2A	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06	Bl35EU
2019_Bl2B	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06	Bl35EU
Bl35EU																	62-15-06	
2019_Bl9A	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	62-21-06	
2019_Bl11A	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-	-	62-24-00	
2019_Bl11B	+	-	+	+	+	+	+	-	-	-	+	+	-	-	+	-	62-24-02	
2019_Bl7B	+	-	+	+	+	+	+	-	+	-	+	(+)	-	-	+	-	62-26-02	
2019_Bl12A	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	+	62-27-06	
2019_Bl12B	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	+	62-27-06	

(+) Indicates slightly reduced sporulation

Core Objective 8 - The development of real-time disease risk monitoring:

We propose to include the AHDB co-funding to develop the B. lactucae *diagnostics component within objective 8*.

Molecular diagnostic tools and markers for *Bremia lactucae*: A recent review 'Advances in diagnostic of downy mildews: lessons learned from other oomycetes and future challenges' Crandall et al (2018) provides the basis for the development and testing of diagnostic assays for *Bremia lactucae* in this project. As a starting point, existing conventional and quantitative PCR assays (Kunjeti et al 2016 and Mota et al 2011) will be tested for their robustness and should provide the basis for gold-standard laboratory testing and for comparison with assays suitable for in-field use. Kunjeti et al (2016) have described the development of a sensitive and specific qPCR-based assay using a target sequence in mitochondrial DNA for the specific detection of *B. lactucae* and its use to detect and quantify airborne *B. lactucae* near lettuce crops, thus providing an estimation of the inoculum load and enabling judicious timing of fungicide applications. The assay was deployed using spore traps in the Salinas Valley, where nearly half of U.S. lettuce is produced and will be useful for quantifying inoculum load in and around the lettuce fields for the purpose of timing fungicide applications based on inoculum load. The assay is not however suitable for direct in-field use. Real-time PCR assays are both sensitive and specific in their ability to detect and quantify target DNA but they require thermocyclers and are better suited to laboratory use. Loop-Mediated Isothermal Amplification (LAMP) is a simple, rapid and specific nucleic acid amplification method. It is characterized by the use of 4 or 6 different primers specifically designed to recognize 6 distinct regions on the target gene and the reaction process proceeds at a constant temperature using strand displacement reaction (Notomi et al., 2000). Amplification and detection of target DNA can be completed in a single step by incubating at a constant temperature.

Isothermal amplification techniques, including LAMP, have the advantage over real-time PCR techniques in that they do not require thermocyclers making them suitable for incorporation in to in-field detection devices.

M8.1 Assay for in-field detection of *B. lactucae*. (M6-12)

Bremia lactucae was isolated from lettuce samples naturally infected with downy mildew. Lettuce leaves with suspected downy mildew lesions were placed on damp paper towel in perspex boxes in the glasshouse until lesions sporulated (usually within 7-8 days). Spores were washed off sporulating leaves and the spore suspension centrifuged to remove debris, and then again to pellet the spores. As *B. lactucae* is an obligate pathogen (non-culturable),

isolates were maintained on universally susceptible lettuce seedlings e.g. cv. Cobham Green. Alternatively, isolates were stored at -20ºC then -80ºC as spore pellets which were then retrieved by thawing and resuspending in water before pipetting a small amount of spore suspension on to moistened lettuce seedlings.

Sporangia of other obligate pathogens e.g. *Peronospora destructor*, *P. meconopsidis* and *P. parasitica* were collected from host plant seedlings, onion, ornamental poppy and column stocks respectively, which had previously been inoculated with sporangia and incubated to induce sporulation. The concentration of sporangial suspensions were measured using an haemocytometer and adjusted to 8000 spores/µl. Suspensions were then freeze-dried in 1.5 ml Eppendorf tubes. Isolates of *Phytophthora* species were grown on rye A agar or pea agar incubated at 18ºC for 7 days and isolates of *Alternaria* and *Pythium* spp. on potato dextrose agar (PDA) incubated at 20°C for 10 days. A 6 mm^2 plug was then taken from the edge of the colony and used to inoculate 20 ml of pea broth (*Phytophthora* spp.) or potato dextrose broth (other spp.) in a sterile 9 cm Petri dish. After 10 days incubation at 18ºC or 20°C respectively the agar plug was discarded, and the resulting mycelium was washed in sterile distilled water and dried on a paper towel. A 0.1 g sample of mycelium was weighed and freeze-dried in a 1.5 ml Eppendorf tube overnight. DNA was extracted from freeze dried sporangia or mycelia using a method modified from Raeder and Broda (1985). DNA concentration (ng/µl) was quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Loughborough, UK). Species identification of pathogens originally isolated from naturally infected host plants was confirmed by testing with PCR, using the assays of Kunjeti et al. (2016), Brouwer et al. (2003) and Landa et al. (2007) for *B. lactucae*, *P. parasitica* and *P. destructor* respectively. All other species were kindly supplied by David Cooke (The James Hutton Institute).

Bremia lactucae LAMP assay primer design

Mitochondrial DNA sequences were BLAST searched and compared with closely related species to find non homologous regions. Primer design was carried out using LAMP Designer (Optigene, Horsham, UK). The six LAMP primers (Blact1); external primers F3 and B3, internal primers FIP and BIP, and loop primers F-loop and B-loop were synthesised by Eurofins Genomics.

Real-time LAMP specificity and sensitivity

Real-time LAMP assays were carried out in a final reaction volume of 25 µl : 15 µl Isothermal Master Mix ISO-001 containing a fluorescent ds-DNA binding dye (Optigene), 5 µl primer mix consisting of external primers (F3 and B3) at 5 µM, internal primers (FIP and BIP) at 20 µM, loop primers (F-loop and B-loop) at 10 µM final concentration and 5 µl DNA. Real-time LAMP

was carried out using a StepOnePlus-System (Applied Biosystems). Reactions were incubated at 65°C for 30 min and the product visualised every 30 s using FAM detection channel.

The specificity of the *B. lactucae* LAMP primers was tested against genomic DNA (10 ng/µl) of thirty-two isolates of *Phytophthora* species from various hosts, three isolates of *Pythium* spp., and three of *Peronospora* spp. and isolates of *A. solani* and *A. alternaria*. To test real-time LAMP assay sensitivity DNA was extracted from suspensions of known spore concentration and a serial dilution of the DNA was made to the equivalent of 10ng, 1ng, 100 pg, 10 pg, 1pg and 0.1 pg *B. lactucae* DNA/reaction. Non-template controls were tested in duplicate using 5 µl HPLC grade water in place of DNA during every run. The detection limit of *B. lactucae* was 100pg DNA/reaction (Figure 19).

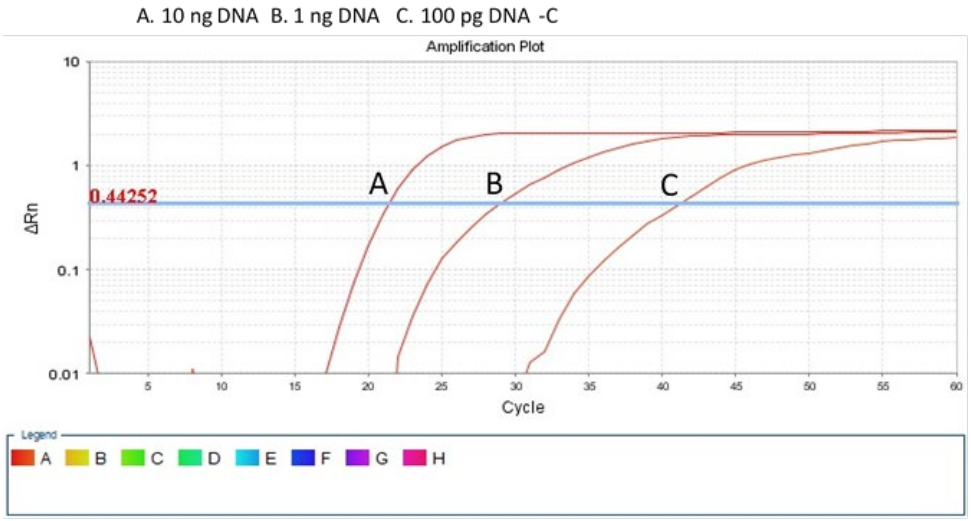

Figure 19. Amplification plot illustrating detection limit (C = 100 pg DNA of *B. lactucae* per reaction) of the LAMP assay, where the threshold cycle is the number of PCR cycles at which the detection of *B. lactucae* DNA exceeds the baseline fluorescence level ΔRn = 0.44.

The specificity of the real-time LAMP Blact1 primers was tested against genomic DNA of 27 *Phytophthora* spp., 3 *Pythium* spp., 2 *Alternaria* spp and 3 *Peronospora* isolates. Only *B. lactucae* DNA was amplified by the Blact1 LAMP assay.

Refining molecular diagnostic approaches to seed testing for viable downy mildew inoculum in sweet basil, spinach, and column stocks – work carried out at NIAB

Background:

Early diagnosis, or better still, actions that prevent downy mildew disease outbreaks before they start are a major benefit to the grower and can help to enhance sustainability in production systems. New outbreaks of downy mildew are reported to be initiated through the sowing of contaminated seed or exposure to oospore-contaminated stem debris in the field for certain species. Once established, the disease can spread rapidly in crops through localised transmission of asexually conidia by wind/rain-splash spread from sporulating lesions. The role of oospores in the transmission of *Peronospora effusa* is well established (Kandel *et al*., 2018), however oospore production in *P. belbahrii* on basil has only been documented in Israel (Cohen *et al*., 2013b; 2017). Recent studies on European *P. belbahrii* isolates found no evidence of oospore production in contaminated seed lots, despite qPCR testing indicating that the disease was present in the seeds of symptomatic samples (Garibaldi *et al*., 2004; Jennings *et al*., 2017). The plating of seed for grow-out testing for obligate pathogens such as downy mildews is ineffective for identifying infections in seed, also detecting downy mildew contamination using conventional grow-on or controlled-environment testing is notoriously inaccurate, with seed-lots exhibiting high-levels of disease in a commercial production setting often showing no symptoms even when thousands of seeds are tested under conditions conducive with symptom-expression. The visual symptoms of downy mildew are relatively simple to identify (Figure 20a & b) but unfortunately by the time symptoms are visible, the quality of the product is likely to have been compromised, and controlling outbreaks can be difficult due to fungicide resistance and adhering to harvest interval and minimum residue requirements. Therefore, the use of DNA-based diagnostic strategies, particularly quantitative PCR and LAMP (Loop mediated isothermal AMPlification), have become standard approaches for detection of downy mildew in high-value crops (Shao & Tian 2018). PCR-based diagnostic strategies have been developed for a number of important pathogenic oomycetes, with quantitative DNA assays designed to detect major downy mildew pathogens on high-value leafy salads (Thines *et al*., 2009; Feng *et al*., 2014; Klosterman *et al*., 2014).

Assays have not yet been developed for detecting either of the proposed DM species infecting column-stock (Jafar, 1963; Koike, 2000), however greater access to cheaper DNA sequencing technology is facilitating the ease at which genetic sequences for designing new molecular diagnostics can be obtained.

DNA-based detection tools can be used to effectively test for downy mildew contamination in tissue, seed and soil (Jennings *et al*., 2017), and commercial tests are available through specialist service providers. Unfortunately when providing specific, quantitative detection of

pathogen DNA, molecular diagnostic tools are unable to distinguish between templates that have come from 'live' and 'dead' cells, limiting their ability to provide accurate estimates on the risk posed from growing infected seed or in contaminated field sites, and linking it with actual symptom development.

Figure 20. a) Sporulating *P. belbharii* on the underside of basil cv. Sweet Genovese; **b)** *P. belbharii* conidiophore (20 x)

For example, a seed lot or soil sample may test positive for downy mildew, however the number of viable propagules present may pose only a very low risk to the grower. Alternately, a seed-lot may have received hot-steam treatment providing effective decontamination, but using conventional DNA diagnostic procedures the sample would still test positive giving a false indication of disease risk.

As an alternative strategy, RNA (*Ribose Nucleic Acid, the signal transcribed from DNA and subsequently translated into protein is less robust than DNA and its presence in quantity can indicate viability*) can be quantified to effectively estimate the amount of a viable organism present in a sample (Wong & Medrano, 2005). The requirement to generate cDNA (copy DNA) from RNA for testing makes the process more expensive and laborious, and has thus far limited the techniques application for commercial seed testing for oomycete pathogens.

A technique termed viability-qPCR has been developed for selectively amplifying the DNA from live and dead cells of bacteria, fungi and yeasts (Alvarez *et al*., 2013; Vesper et al., 2008; Andorra et al., 2010). The strategy utilises a photo-reactive dye called prodium monoazide (PMA) which can bind covalently to DNA on exposure to light, inhibiting PCR amplification, increasing the number of cycles it takes to detect a target compared to an

untreated sample. PMA is incapable of passing through live cell-walls and can only bind to the DNA of dead cells where the integrity of the cell wall has been compromised. Thus there should be no difference in the number of PCR amplification cycles required to detect the DNA of live cells, compared to an increase in samples containing dead cells which have up-taken the PMA. Comparison of PMA treated and untreated live and dead cell-culture standards can be used as reference to compare to the amount of DNA detected in biological samples, providing the operator with an insight into the number of viable propagules.

This technique has not been applied widely to fungal or oomycete pathogens, however the PMA should not be able to penetrate live cell-walls of DM conidia or oospores in the same manner as bacterial cells, but still bind to DNA when the cell wall is disrupted in dead or damaged fungal propagules. Therefore this could be used to provide a more accurate strategy to determine the levels of viable pathogen contamination in a sample and the risk these may pose to the grower in order to improve strategies to prevent further outbreaks of downy mildew in high value herbs and ornamental species.

Materials and methods

Quantification of spore suspensions

Conidial suspensions of *Pb, Pe* and *Peronospora matthiolae (Pm)* (1 x 10^5) for extracting DNA from were quantified in triplicate using a haemocytometer visualised on an Olympus BN2 microscope at 20 x magnification.

DNA extraction

DNA extraction was 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.

Conventional PCR on Hyaloperonospora parasitica/Peronospora matthiolae

PCR was conducted on an MJ Research thermocycler, using 10 µl reaction volumes using primers Hp-Lgsub-F: 5'CACGAATTTATTTGTGCGGCG and Hp-Lgsub-R: 5'-GGGTACCAACATATGTGCTCAA. Amplification products were resolved on a 1% agarose gel run for 45-60 mins at 50 volts and were sequenced by an external service provider (Genewizz, UK)

Quantitative PCR

Extracted DNA was analysed on an ABI Step one plus in a 96-well format using 10 ul volumes with triplicate technical replicates per sample. Assays for detecting *P. belbahrii* (BAS-F1 5'-CCGTATCAACCCAATAATTTGGGGGTTAAT, BAS-R1: 5'-TTACAATCGTAGCTACTTGTTCAGACAAAG[7]; PbITS2-F: 5'- CTGAACAGGCGCTGATTG, PbITS2-R: GCAACAGCAAAGCCAATTC[6]; *P. effusa:* PfsITSF: GTTCGATTCGCGGTATGATT, PfsITSR: TCACACAGCAAAGCCAATTC[8]; were validated on pure DNA extracted from spores over a 10 ng-100 fg/ul 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 was 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.

PMA-PCR on bacterial cells and oomycete spore-suspensions

PMA PCR kits and plate illuminator where purchased from Biotium (Fisher Scientific, UK). Samples of *E. coli* cells were prepared from over-night cultures maintained at 37°C and *P. belbahrii* spores (1 x 10^5/ml) were prepared by washing the leaves of freshly sporulating plants. The respective cell-suspensions were then divided into equal volumes to provide identical samples for live/dead PMA treatments, and non-PMA treated samples for comparison. Bacterial cells and downy mildew spores in one sub-sample were inactivated by heating at 90°C for 5 minutes. PMA treatment and PCR were conducting according to the manufacturer's protocol. PMA treated samples were illuminated for >15 minutes to ensure activation of the dye. Bacterial DNA was extracted using an Ultrapure DNA kit whereas oomycete DNA was extracted using a DNEasy (both from Qiagen UK). Standard dilution series were prepared from PMA-free live and dead cells in order to accurately assess potential differences in DNA content due to heat-inactivation. DNA extracted from live/dead and PMA-treated live/dead samples were analysed using quantitative PCR as described above.

Live and Dead cell dCT determination

In order to accurately determine the percentage of live cells in a sample and quantify the amount of DNA present the dCt (delta Ct: difference in Ct between treated and untreated samples) of live cells is subtracted from the dCt from the deads cells. It is necessary to compare Ct values for each of the four different treatments (i.e. Live cells/+PMA, Live cells/-

PMA, Dead cells/+PMA, Dead cells/-PMA) due to potential variation caused by adding PMA and denaturing the cells. dCts for live and dead cell samples are calculated as follows:

dCt live = Ct(live, PMA-treated)-Ct(live, untreated)

dCt dead = Ct(dead, PMA-treated)-Ct(dead, untreated)

The live treated/untreated components should have a dCt = 1+/- (meaning PMA treatment should not increase by >1+/-). Once the dCts for live and dead cell samples have obtained the change in dCt can be calculated to provide a quantitative estimation of the quanity of live cells in a sample.

The difference between dCt live and dCt dead must be >4 to be considered significant. As qPCR amplifies DNA on a log scale, a difference in dCt 4 is equivalent to a 16-fold decrease, or 94% dead cells in a sample.

If the live and dead cell controls are in the correct range, the percentage of viable cells in unknown samples can then be estimated by firstly calculating the dCt for the unknown samples as shown: dCtsample = Ct(sample, PMA-treated) - Ct(sample, untreated)

The dCt can them be converted into a percentage of viable cells as shown:

Fold reduced by PMA = 2(sample dCt) % viable = 100 / Fold reduced.

Conventional grow-on tests

A minimum of 3000 seed were screened for each seed sample chosen. Spinach and basil seed were sown in batches of 100 seed grown until plants were at the fourth true leaf stage. Plants were regularly wetted, placed in high humidity condition overnight and assessed for the appearance of downy mildew symptoms.

Box tests

Basil seed were sown in 7 x 7cm seed trays (15 seed/pot). The compost wet thoroughly, covered with a propagator and placed in a growth chamber at 19-24°C (16 h light) until seedlings reached the 1st true leaf stage (13-15 days). Seedling were cut at the stem base and placed inverted onto wet filter paper in Perspex boxes. Lids were placed on the boxes and the seedlings incubated for 24 h in a dark growth chamber and then maintained at 19-24°C with a 16:8 h light/dark for a further 8 days.

Assessments for oospore production in inoculated plants

No oospores have been observed in mature infected basil or column-stock cultivated under growth room conditions during the study to date. Inoculated spinach plants for providing

oospore contaminated leaf/stem material for optimising extraction and viability testing are currently being cultivated.

Results

Development and optimisation of oospore viability determination qPCR methodologies

Design/optimisation of oospore extraction and Taqman qPCR assays for P. belbahrii/H. parasitica, (testing of P.farinosa fsp *spinaciae/P. effusa)*

Screening of qPCR assays

Quantitative PCR assays for specific detection of Pb and Pe are documented in the literature (Table 21). The respective assays were selected for validation, testing for specificity and performance against purified DNA templates to ensure suitability for subsequent use in the viability determination assays, however no assay could be identified to effectively detect downy mildew on *Matthiola incana.* For *Pb,* two SYBR Green type assays had been utilised for previous basil seed testing applications; both of the assays were observed to be specific, demonstrating suitable amplification profiles and thus suitable for viability-qPCR applications. For *Pe,* SYBR green and Taqman type assays were selected for screening; the SYBR Green assay was observed to perform favourably whereas no amplification products could be generated using the Taqman assay, rendering it unsuitable for further assessment. The *Pb* and *Pe* SYBR green assays could accurately detect target DM DNA over a range of 10 ng/ul to 100 fg/ul, enabling even low amounts of pathogen DNA to be detected in contaminated samples.

Table 21. *Pb* (*P. belbahrii*) and *Pe* (*P. effusa*) specific qPCR assays tested

Species.	Assay type	Designer	Amplifies specific target DNA	Cycle threshold ($1x10^5$/ml)
P. belbahrii	SYBR Green	Thines *et al.,* 2009	Yes	11
P. belbahrii	SYBR Green	Shao & Tian, 2018	Yes	14
P. effusa	Taqman	Klosterman *et al.,* 2014	No	(*)
P. effusa	(SYBR Green)	Feng *et al.,* 2014	Yes	7
P. matthiolae	N/A	N/A	N/A	(*)

No oospores have been observed in infected basil or column-stock produced under field or growth room conditions in the current or recent (AHDB report PE 024) screening. Symptomatic spinach plants inoculated with *P. effusa* for collecting oospores for optimising extraction and subsequent viability testing are being grown to maturity. Homogenisation strategies for plant-material and seeds will now be optimised for obtaining oospores.

Genetic characterisation of downy mildew isolated from M. incana (basic diversity assessment, SSRs, MinION run)

There are currently no suitable assays for detecting either *Hyaloperonospora parasitica* or *Peronospora matthiolae* affecting *M. incana.* In an effort to confirm which species of downy mildew had been isolated from column-stock in the UK, we attempted to sequence the internal transcribed spacer (ITS) region with universal fungal/oomycete primers (ITS1, 4). It was not possible to generate any amplification products, despite manipulating the PCR conditions to decrease specificity. In order to further try and confirm if the identity of the isolate, new oligonucleotide primers were designed in select genes in *H. parasitica* (EF-1, G3PD) using sequences obtained from public repositories (Genbank). As previously the assays were unable to detect the correct target effectively. Another primer pair was then designed in the large ribosomal subunit (rRNA) which successfully amplified a region of DNA from the target DM and the fragment was subsequently sequenced. Comparison of the fragments sequence revealed a high level of similarity (approx. 97% across 497/2307 bp, 21% of its length) with other closely related spp. including *Hyaloperonospora arabidopsidis, H. parasitica, H. erophilae* and *H. thlaspeos-perfoliati.*

No sequences could be identified for the *P. matthiolae* ITS region in the sequence databases or any with 100% similarity. Lesions of the putative *P. matthiolae* isolate were characteristically white and conidiophores irregularly clumped compared to the grey, fluffy appearance of *P. belbahrii* and dense purple-blue lesions observed for *P. effusa.* The lesions where most similar in appearance to downy mildew of *Arabidopsis thaliana* or those described for *P. matthiola*[12]. Microscopic assessment of *P. matthiolae* conidia observed slightly ovoid conidia with a width of approx. 21 µM and length of approx. 25 µM (Figure 21). The lack of genetic sequence data for the DM isolated from *M. incana* hampered effective assay design and therefore high molecular weight DNA (1 µg) was extracted from the *P. matthiolae* and sent to an external service provider for sequencing for generating the raw data for assembling a draft genome and designing specific diagnostic assays.

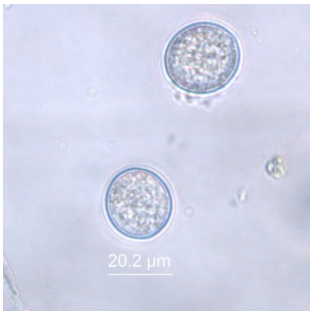

Figure 21. Conidia isolated from symptomatic column-stock (40x)

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.

Viability qPCR methods were tested on *Escherichia coli* cell cultures and applied to freshly harvested *Pb* conidia to assess the performance for quantifying live pathogen cells in biological samples. The PMA-qPCR procedure assess for differences in the qPCR Ct value for live and dead cells treated with PMA dye, and then quantified against a standard generated on pure pathogen DNA. Preliminary assessments were conducted with standardised titrations of *E. coli* cells to establish the limit of detection and optimise PMA dye photo-excitation treatments. The manufacturer's protocol indicated an exposure duration of >15 min on the illumination-box; no effect from the PMA treatment was observed with shorter durations. After treatment with PMA and photo-excitation, the samples containing the dead, heat-shocked cells were visibly darker red in colouration compared to the live/control samples indication the PMA dye had bound to the DNA (Figure 22).

Figure 22. 1.5 ml micro-centrifuge tubes containing PMA untreated/treated cells

Templates were extracted from *E. coli* and *Pb,* respectively and analysed using qPCR. On average, the difference in Ct between live and dead PMA-treated cells *E. coli* averaged 6.4 cycles (Table 22), with more than 95% of the DNA neutralised from the dead cells. This demonstrated how treatment with PMA dye and illumination delayed the onset of PCR in the dead samples. In addition to the difference in Ct between live, dead and PMA treated cells, assessment of control reactions demonstrated how killing cells with the heat-treatment resulted in a reduction in Ct of approx. 2 in *E. coli.* This highlights why the inclusion of a standard dilution series from DNA extracted from dead cells is recommended by the PMA kit manufacturer and how differences in Ct must be accounted for when estimating the concentration of DNA in the different types of sample.

Table 22. PMA-qPCR results from testing *E. coli* cells

E. coli sample	Ave. Ct
DNA Standard (1x10_5)	11.2
PMA treated live cells (1x10_5)	11
Dead cells (1x10_5)	13
PMA treated dead cells (1x10_5)	19.4
dCT live	**(-0.2)**
dCT dead	**(6.2)**

The procedure was then trialled on *P. belbahrii* conidia to gauge if denaturation and PMA treatment could be applied to downy mildew propagules. The process of heat-killing and PMA exposure was implemented in *Pb* cells in the same manner as *E. coli,* with the only deviation that the DNA extraction methodology was tailored for plant/fungal templates. The average difference between live and dead PMA treated *Pb* conidia was approx. 1.8 dCT based on two independent experiments (Table 23). A reduction in Ct of approx. 4 cycles was also observed between live and dead samples, highlighting how killing of the spores resulted in a reduction in the quantity of DNA extracted; this was observed in *E. coli* however a greater quantity of DNA was retrieved than for *Pb*. This exemplifies why it was necessary to include a standard for heat inactivated cells but also that the extraction procedure for dead *Pb* spores may require further optimisation. The PMA-qPCR strategy will now be applied to *Pe* conidia and oospores, and adapted to test the approach's suitability for seed testing.

Table 23. PMA-qPCR results from testing *Pb* spores

P. belbahrii run 1	**Ave. Ct**
DNA Standard (Live spores, 1x10_5)	14.2
PMA treated live spores (1x10_5)	15.4
Dead spores (1x10_5)	18.2
PMA treated dead spores (1x10_5)	21
dCT live	**(1.2)**
dCT dead	**(2.8)**
P. belbahrii run 2	**Ave. Ct**
DNA Standard (Live spores, 1x10_5)	14.2
PMA treated live spores (1x10_5)	15.2
Dead spores (1x10_5)	17.62
PMA treated dead spores (1x10_5)	20.73
dCT live	**1**
dCT dead	**(3.02)**

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

- *Optimisation of seed lot screening strategies to establish validated sampling protocol/*
- *Testing of up to 20 x suspect Spinach/Basil/M. incana seed lots (from industry sources) for the presence and viability of DM oospores*

A total of 10 seed lots have been obtained for testing for DM contamination to date (Table 24). These have been sub-sampled in batches of 3,000 seed and are now being tested in bulks of 50, 100, 500 and 1000 seed for the presence of *P. effusa/P. belbahrii* the validated molecular assays (see above) in order to gauge sensitivity, establish the limit of detection and improve understanding of the incidence of downy mildew in seed lots. PCR testing of ethanol-washed seeds will first be conducted in order to assess for external contamination in debris, followed by drying and homogenisation of seed for releasing oospores/accessing internal contamination and extracting RNA/DNA for performing the diagnostic tests. Conventional qPCR will be used confirm the presence of DNA, and any samples testing positive will be subsequently tested for viability. There is resource available in the project to undertake testing on a further 10 seed lots (20 in total).

Table 24. Seed lots for testing in CP 184

Coded seed-lot	Species	Origin	Steam Treated
CP 1	Basil	CP 184	N
CP 2	Basil	CP 184	Y
CP 3	Basil	CP 184	N
CP 4	Basil	CP 184	Y
CP 5	Basil	(PE 024)	N
CP 6	Basil	(PE 024)	N
CP 7	Spinach	CP 184	N
CP 8	Spinach	CP 184	N
CP 9	Spinach	CP 184	N
CP 10	Spinach	CP 184	N

Screening and testing of commercial seed lots using grow-on testing under indoor/outdoor conditions to validate new assays, observation of disease outbreaks from tested seed under production.

Establish grow-on tests under protected conditions for contaminated seed lots of M. incana/ O. basilicum /S. oleracea

Grow-on tests have been used to screen Spinach lots CP 8 and CP 9 3 x 1000 seed per batch), however no diseases symptoms have been observed.

Box-tests have been conducted for three basil seed lots (CP 1, 3 and 5, Table 25) and again no downy mildew symptoms have been observed..

Table 25. Seed-lots selected (so far) for box-tests

Seed sample	Numbers of seed tested	Symptomatic seedlings	Infection (%)
CP1	500	0	0
CP3	500	0	0
CP5	500	0	0

Discussion

A range of quantitative DNA assays for *Pb* and *Pe* have been published in the scientific literature, however, it was necessary to assess if the assays on offer were specific in their ability to identify their targets, including all different race-types for *Pe*. Both of the *P. belbahrii* assays tested detected the DNA template in a specific and reproducible manner, in agreement with outputs observed for previous basil seed testing efforts.

The Taqman assay designed for *P. effusa*[9] failed to amplify DNA templates despite repeat attempts, and further efforts, to reduce annealing temperatures to promote primer binding. This indicates that the assay is unsuitable for detecting all *P. effusa* races, particularly the race-8 type used for this study. The failure of the *Pe* Taqman assay likely represents a difference in genetic sequence at one of the primer or probe sites. It is not uncommon to experience genetic variation between different races of DM and even if efforts have been made to select for all pathotypes it is still possible to miss specific mutations; in this case the assay does not detect the race type 8 used in the experiments.

Despite the close similarity between the target and the related species, failure of other PCR reactions suggest the downy mildew isolated from column-stock is likely to be a separate organism to *Hyaloperonospora parasitica* (syn. *Peronospora parasitica*). This is also supported through visual observations of reproductive structures and conidia isolated from infected plant-material which resemble those described by Jafar *et al.* (1963), but also Koike *et al.* (2000). In order to verify this hypothesis DNA extracts from *P. matthiola* have been collected from inoculated column-stock and are currently being bulked in isolation to obtain DNA templates for conducting whole-genome sequencing, assembly and subsequent design of an effective diagnostic PCR assay.

Comparison of sequences in the large ribosomal subunit revealed a high level of similarity (>95% across 21% length of the sequence) with other closely related spp. including *Hyaloperonospora arabidopsidis, H. parasitica, H. erophilae and H. thlaspeos-perfoliati*. The observed high levels of similarity with closely related downy mildew species were anticipated and this is a common occurrence when comparing rRNA sequences. However further analysis was limited by a paucity of sequence data for *P. matthiola* preventing comparisons with other species including *H. parasitica.* Sequencing of *P. matthiola* is currently underway and this will provide essential genetic information that will be used to design discriminatory diagnostic assays to improve detection and control of downy mildew on column-stock, and broaden our fundamental understanding of how the genome of this important pathogen is organised.

PMA-qPCR was trialled as a strategy to estimate propagule viability in a fixed concentration of *E. coli* cells (1 OD) and *P. belbahrii* conidial suspensions (1 x $10^{5/}$ml). The respective assays could accurately and reproducibly detect their target DNA and PMA treatment resulted in a substantial increase in dCT in dead compared to live cells from *E. coli* (6 dCT), with a smaller difference observed for *P. belbahrii* (1.81 dCt). Whilst below the threshold for accurate determination in *Pb* (dCT>4), this demonstrated that the technique has potential for optimisation in downy mildew species. The findings also indicate there is potentially less DNA or that it is harder to extract DNA effectively from dead spores compared to the live spores alone, with a lower amount of DNA present in dead *Pb* samples. This was not the case when extracting DNA from dead *E. coli* cells and could indicate that the extraction procedure for *Pb* is not optimal. As a reduction in DNA was also observed for the PMA treated live spores, it is suggested that PMA treatment may negatively influence the extraction process, leading to a lower DNA content, however this is generally observed to be within the 1+/- dCT limit advised by the kits manufacturer. Thus alternate DNA extraction methods and extended heat exposure duration to ensure all cells are killed effectively will be trialled in conjunction with parallel testing of additional PCR assays[9] in an attempt to increase accuracy.

This is potentially the first occasion that this type of approach has been applied to downy mildew affecting high value leafy herbs. This work has demonstrated how the PMA-qPCR technique could have potential for contributing to improving estimation of pathogen viability in biological samples. The technique is now being used to test a dilution series in *Pb* to assess assay sensitivity and the limit of detection in the terms of both DNA concentration and spore number (1 – 1 x10^5/ml). Assays will also be run on *Pe* and *Pm* conidia (as soon as a diagnostic is available for *Pm),* on extracted *Pe* oospores, and on basil and spinach seeds contaminated with DM.

A total of six basil seed lots and four spinach seed lots have been procured for testing in the project so far, with sufficient resources to test a further ten suspect samples from the three target crops. Seed lots are currently being sub-sampled for washing and PCR testing to assess for external contamination before proceeding to test seed extracts and trial PMA-qPCR and RT-PCR approaches. Grown-on testing and detached seedling box tests are currently being implemented for three basil seed lots, with the remainder of lots scheduled for screening in the coming months.

Conclusions

- More research is needed on elicitors, their interactions with specific pathosystems have to be further explored in time and space to maximise reliability and efficacy. Also, the impact that natural elicitors from various stresses have on crops impacts efficacy of applied materials (Walters *et al.,* 2013) – it is still uncertain whether elicitor applications to outdoor crops provide consistent economic benefit when used on outdoor soil grown crops exposed to natural elicitors.
- Seaweed extracts benefit the plants in various ways, these benefits are small but can easily be used to help improve overall plant health. Phosphite has recently been registered in the EU as an active ingredient for plant protection having shown efficacy against oomycetes. It is still currently available as a component in many products that are sold as fertilisers or biostimulants, not as plant protection products. Chitin is another product that shows great promise as an elicitor, it doesn't help to improve nutrient uptake and as such will not be able to be included into the new EU fertiliser laws which cover biostimulants. Interestingly AMF have proven potential stimulating plant defences against soilborne pathogens, but their use against aerial oomycetes has not been explored.
- DNA can be detected specifically for *Peronospora belbahrii* and *Peronospora effusa* in plant and seed samples using published diagnostic assays.
- Viability qPCR strategies developed for bacterial targets have the potential to be adapted to work effectively in oomycetes
- Downy mildew isolated from Column stock in the UK is likely to be *P. matthiola* based on initial morphological and genetic testing.
- Published information has been collated on *Bremia* populations and isolates have been collected from UK growers (ongoing process). The first 15 isolates have been race tested against IBEB differentials and 11 putative races have been identified
- LAMP primers have been designed for *Bremia* and have so far been successfully tested for specificity and efficacy in LAMP qPCR (ongoing).
- The first year's fungicide sensitivity assays have been successfully completed for *P. parasitica* and *Bremia lactucae* isolate collections. Results so far are reasonably consistent with expectations and fungicide concentration ranges selected for assays successfully provide suitable ranges for calculation of ED_{50} values which will be useful in assessing trends in observed sensitivity in pathogen populations over time (and with location of isolation?).

- Review of general downy mildew biology and of DST for their control in selected key horticultural crops shows that a range of options are currently available, from zero through simple risk rules to sophisticated simulations and forecast models as well as new possibilities of affordable molecular inoculum detection to further refine precision of risk assessments. Combinations of forecasts and inoculum detection are improving the accuracy and potentially the timing of risk warnings which is important as the range of fungicides available for control of downy mildews are predominantly of protectant action with the few curative chemistries available carrying medium to high fungicide resistance risks.
- Review also indicates the possibility of applying simple rules-based DST or even some adapted forecast models to minor crops could be effective at supporting IPM and that the main influences on uptake of DST in general where the perceived high risks and more importantly the degree of 'user-friendliness' of the operational front ends of systems.

Knowledge and Technology Transfer

- Poster presented on seed-borne infection/diagnostics work at NIAB Annual Science day & Director's day 2019
- Oral presentation summarising entire project 27 Feb 2019 to Leafy Salads Group, Evesham.

Glossary

Term	Definition
Arbuscular mycorrhizal fungi	Common type of endomycorrhizal fungus that forms a symbiotic association with plant roots.
Ascophyllum nodosum (L.)	A species of seaweed, commonly used in production of seaweed extracts for biostimulant products.
Biotrophic	It is a pathogen or parasite that feeds on the hosts and requires living cells to feed
Biostimulant	A material that contains substance(s) and/or microorganisms whose function, when applied to plants or the rhizosphere, is to stimulate natural processes to benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and/or crop quality, independent of its nutrient content
Chitin	An abundant natural polysaccharide, chitin can be found in a wide range of organisms, most notably exoskeletons of arthropods (e.g. crustaceans and insects) and the cell walls of fungi
Chitosan	De-acetylated form of chitin (poly(D-glucosamine)
Complex organic materials	Broad range of products that contain material derived from the remains of organisms (e.g. plants).
Elicitor	In plant pathology, a compound that induces a defence response to damage or infection in the plant. Can be biological or chemical in origin.
Fulvic acids	Extraction product of humus
Humic substances	Extraction product of humus
Nematodes	Round, unsegmented minute wormlike animals
Necrotrophic	A pathogen that kills host cells then feeds on the dead cells
Non-essential chemical elements	Elements that are not necessarily required by all plants but can promote plant growth
Non-pathogenic fungi	A wide range of fungal species that have no direct pathogenic effect on plants
PGPR	Plant growth promoting rhizobacteria
Plant growth promoting rhizobacteria	Bacteria that inhabit the rhizosphere, which have been shown to benefit the plant growth
Rhizobium	A genus of common nitrogen fixing bacteria. Form nodules in leguminous plants (e.g. peas and beans) to establish a symbiotic relationship, providing nitrogen to the plant in exchange for carbon.
Rhizosphere	Volume of soil influenced by plant roots
Seaweed extract	Products that have been extracted from seaweed via either a chemical or natural extraction process
Systemic acquired resistance	Whole plant resistance response to localised exposure to a pathogen or certain chemicals
Induced systemic resistance	Localised interactions with some plant growth promoting rhizobacteria results in plant becoming resistant to some pathogenic bacteria, fungi and viruses.

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