Project title:	Downy Mildew & Late Blight Control
Project number:	CP 184
Project leader:	Tim Pettitt, Eden Project Learning (EPL) Alison Lees, (JHI) Tom Wood, (NIAB) Erika Wedgwood, Year 1 (RSK ADAS Ltd)
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Key staff:	Alison Lees, Tom Wood, David Cooke, Jane Thomas, James Lynott, Simon McAdam, Ann Webb, Beatrice Corsi, Erika Wedgwood, Tim Pettitt.
Location of project:	EPL, Bodelva, Cornwall; JHI, Invergowrie Dundee; NIAB, Cambridge & RSK ADAS Boxworth, Cambridgeshire (Year 1)
Industry Representative:	Emma Garfield, R & D Agronomist and Head of Technical, G's Growers, Barway, Ely, Cambridgeshire
Date project commenced:	January, 2019

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AUTHENTICATION

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

[Name]

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[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

Integrated management is now essential for the effective management of downy mildews on horticultural crops. Reported here is the final year of work on an ambitious project with several lines of investigation bringing together:

1) The development of new molecular tests to detect and quantify downy mildew pathogens in basil, spinach and column stock seed-lots;

2) Heat treatments were demonstrated to be effective at removing infections from contaminated seeds;

3) PMA-qPCR was 'road-tested' for testing seed for viable low-level downy mildew infections – whilst it works, it is very involved and only currently suitable as a very specialised laboratory method for detecting and quantifying low level infections;

4) Lettuce downy mildew race typing and fungicide resistance testing;

5) Updated information about the resistance of lettuce, spinach and column stock downy mildew populations to approved and widely used fungicides;

6) Reviews on the general biology and control of 14 horticulturally important downy mildews, the potential for using elicitors in integrated control and the current state of development of Decision Support Systems/Tools for the management of downy mildews 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'Niell *et al*., 2002), increase the efficacy of chemicals and plant resistance in integrated management programs. Similarly, the use of rapid pathogen detection and disease simulation models can optimise the timing of fungicide applications and in some seasons reduce their number – increasing efficacy whilst reducing costs and potential environmental impacts.

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

Integrated pest and disease management (IPM) is an increasingly important and pertinent area of research for horticulture and this project aimed, through provision reviews and where possible new best grower practice information, consolidated current knowledge, ensuring that measures that can be taken up were quickly disseminated and potential barriers to uptake identified. The project built on current knowledge of several pathosystems (specifically downy mildew on lettuce, spinach and basil) 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, were developed to 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 focussed 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 final year's progress, assessing pathogen races and progress with assessing molecular procedures for determining levels of viable downy mildew present in contaminated seed lots.

Summary

This project 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 (AHDB CP184 report 2019), b) developing and improving detection diagnostic procedures to screen seed for infection to help cut this significant source of disease (AHDB CP184 reports 2019, 2020 and here), 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 (AHDB CP184 reports 2019, 2020 and here), and d) developing fungicide sensitivity test protocols to check pathogen populations for fungicide resistance (AHDB CP184 report 2019). The overall conclusions and findings of the project can be summarised as follows:

- Review has shown that 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 provide consistent economic benefit when used on outdoor soil-grown crops exposed to natural elicitors (AHDB CP184 report 2019).
- 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, although 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 (AHDB CP184 report 2019).

- Molecular testing was demonstrated capable of detecting and quantifying small amounts of basil, spinach and column stock downy mildew (DNA/RNA) both externally (in basil and spinach seed-lots) and inside contaminated seeds of basil, spinach and stocks (AHDB CP184 reports 2019, 2020 and here).
- LAMP primers have been designed for *Bremia* and have been successfully tested for specificity and efficacy in LAMP qPCR (AHDB CP184 report 2019).
- Seed-lots of basil, spinach and column stocks containing very low levels of downy mildew (DNA/RNA) still potentially pose a high disease risk to growers (AHDB CP184 reports 2019 and 2020 for basil and spinach, and here for column stocks).
- Across the seed-lots of basil and spinach tested, a greater quantity of downy mildew DNA was detected inside the seed coat compared to seed washings (AHDB CP184 report 2020).
- Steam-treated basil seed lots contained approximately 50% less downy mildew DNA than untreated samples from identical lots, indicating that steam-treatment reduces the pathogen load (AHDB CP184 report 2020).
- The downy mildew isolated from the column stock samples tested in this study was confirmed as *Pernospora matthiolae* by both morphological characters, and nucleotide sequence analysis, and basic phylogenetic study showed this to be more closely related to *Hyaloperonospora arabidopsidis* than *H. parasitica/H. brassicae* (AHDB CP184 report here).
- Quantitative PCR was conducted on commercial *Matthiola incana* seed lots suspected of containing downy mildew contamination, using a novel assay designed for the project. This is the first time a diagnostic assay has been described for the detection of *P. matthiolae* in seed (AHDB CP184 report here).
- Thirty-nine lettuce *Bremia lactucae* isolates were collected from 2019-2021 and assessed for race structure according to IBEB guidelines and protocols kindly supplied by Naktuinbouw, who also supplied seed of the 16 current accessions in the official lettuce differential set (Set C). Twenty-eight putative races were identified and of these, one (2020_BL4G) matched IBEB committee race description Bl:24EU, whilst four others (2019_BL2A, 2019_BL2B, 2021_BL11A & 2021_BL11C) matched IBEB race Bl:35EU.

Comparisons of this data with publicly available data will continue after the end of this project, whilst race testing results for all of the isolates tested from 2019 to 2021 were included in the IBEB EU Groslist 2021 (AHDB CP184 reports 2019, 2020 and here).

- Viability qPCR using Propidium Monoazide PMA has been demonstrated to be effective in detecting differences in the amount of DNA in live and dead cells from *P. belbahrii* and *P. effusa*. This procedure can be used effectively to determine the presence of viable/no-viable cells of *P. belbahrii* or *P effusa* in infected seed-lots, although research in this project as shown that control assays are needed to account for the internal effects of heat and of PMA addition which require the generation and use of live spores for every test, necessitating a lot more preparation than originally anticipated, and therefore higher costs when compared to reverse transcription qPCR methods that target RNA/active gene expression (AHDB CP184 reports 2020 and here).
- Fungicide resistance tests at JHI were restricted by the availability of fresh isolates from current epidemics. Nevertheless, tests were successfully carried out in 2019 and 2021 on *B. lactucae* isolates from lettuce, and on *P. matthiolae* from column stocks and in 2021 on *P. effusa* isolates from spinach (AHDB CP184 reports 2019 and here).
    - Fungicide resistance tests on *B. lactucae* isolates obtained from the industry in 2019 and 2021 showed that:
        - Mandipropamid (Revus) gave consistently high levels of disease control at field rate (99-100%) across all isolates tested.
        - Dimethomorph (95-100%) and Azoxystrobin (90-100%) also showed good control of lettuce downy mildew at field application rates.
        - These tests were carried out for the calculation of EC50 values for longer-term reference and use alongside pathogen race identifications, some large differences in the min and max values were noted for some isolates, particularly for Dimethomorph and Azoxystrobin. These individual values need to be treated with caution – their true value/meaning will only emerge with ongoing longer-term screening and EC50 calculations.
    - Fungicide resistance testing carried out on *P. matthiolae* isolates by JHI in 2019 and 2021 had the following outcomes:
        - Widespread resistance to Metalaxyl was found across a range of isolates in 2019 with no further testing therefore carried out
        - Fosetyl-Aluminium gave moderate disease control (28-68%) in 2019 and 2021

        - Mandiprompamid gave better control in 2019 (80-86%) than 2021 (52-82%)
        - The tests suggest resistance to Dimepthomorph may be developing given the 80-90% control found in 2019 dropped to 10-15% in 2021
        - Amectotradin/Dimethomorph and Mancozeb gave moderate and variable control of 34-66% and 15-65% respectively in 2021
    - Fungicide resistance testing at JHI on P. effusa isolates in 2021 showed that:
        - When applied at field rate, Mandipropamid and Dimethomorph gave 100% control
        - Azoxystrobin applied at field rate gave excellent control between 99-100%.
        - Calculation of EC50 showed small differences between isolates' reactions to all 3 products tested but these differences were not shown to be statistically significant, showing control in the small population of P effusa tested so far to be consistent.

- Review of general downy mildew biology and of Decision Support Tools (DST) to assist for their integrated management 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 (AHDB CP184 report 2019).
- 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 (AHDB CP184 report 2019).

Financial Benefits

Aerial oomycete infections significantly reduce crop yield, with those affecting plants in propagation, in particular, able to cause total crop loss, and those in ornamentals potentially causing the crop to become unmarketable (Wedgwood, *et al.,* 2016). Timely intelligence concerning prevalent phenotypes present in downy mildew populations has potential for

significant financial benefits in terms both of managing fungicide resistance and the deployment of cultivars with suitable resistance genes. In addition, the detection and interception/treatment of infected seeds is likely to have a large impact on downy mildew incidence in crops such as basil, whilst the effective use of cultural controls and decision support systems could both reduce the frequency of spray applications, improve their efficacy and reduce the pressure selecting for new pathogen genotypes with fungicide resistance and/or capable of overcoming cultivar resistances.

Action Points

- Keep vigilant for the appearance of fungicide resistance and avoid use of metalaxyl for mildew control in column stocks
- Use Fosetyl-Aluminium and Amectotradin/Dimethomorph with caution especially in column stocks and as part of a spray program ‘ringing the changes’ with products such as Mandipropamid
- Consider use of Decision Supports to help with development of integrated control programs for downy mildew even if these are just based on simple ‘rules of thumb’
- Read the review summaries for basil, column stock, lettuce and spinach downy mildews for insights into how to update IPM strategies
- Consider requesting steam-treated seed to help manage the risk of infections starting from seed in basil
- Following the foundation work at James Hutton Institute from this project, race testing will be delivered commercially via an industry consortium and the genotypic profile of *Bremia* isolates could be monitored using the tools developed. This information will inform lettuce variety selection for more effective control of downy mildews. Contact JHI for further information on latest developments and testing possibilities..

SCIENCE SECTION

General Introduction

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

Diseases caused by aerial oomycetes typically exhibit rapid epidemics, which if left unchecked under optimal environmental conditions have the potential to cause complete crop loss either directly by mortality, or by rendering foliar and fruit produce unmarketable. Disease control options are limited or under-utilised and currently management is heavily reliant upon the use of fungicides, often used prophylactically, as none of the available chemicals can reliably achieve curative control, and once disease is observable in crops it will often already have become established and difficult to manage. Unfortunately, the number of currently available fungicides is becoming very restricted as a result of product withdrawals and too few new introductions. The resulting reduction in the number of active ingredients being used in control programs greatly increases the risk of pathogen populations developing fungicide resistance. The use of resistant varieties, where available, is a good disease management option although their use puts huge selection pressure on oomycete pathogen populations for new races capable of overcoming host resistance. Cultural disease management methods (e.g. appropriate tilth management, removal/treatment of crop debris, manipulation of environmental conditions), often have a limited impact on disease when used alone but can greatly (even synergistically, e.g. control of {a non-oomycete with analogous epidemiology} *Botrytis* grey mould in ornamentals, O'Neill *et al*., 2002)~~,~~ increase the efficacy of chemicals and plant resistance in integrated management programs. Similarly, the use of rapid pathogen detection and disease simulation models can optimise the timing of fungicide applications and in some seasons reduce their number – increasing efficacy whilst reducing costs and potential environmental impacts.

The use of contaminated seeds is considered responsible for many outbreaks of downy mildews on basil and spinach, caused by *Peronospora belbahrii (Pb)* and *Peronospora effusa (Pe),* respectively. *Pe* is a seed-borne pathogen, producing heterothallic oospores in the seed coat (Kandel *et al.,* 2019) that cause systemic infection in the crop. The transmission of *Pb*

on or in seed is less clear; only a single case of oospore production has been reported in basil (Cohen *et al.,* 2017) and most new disease outbreaks have been attributed to asexual aerial conidia and become evident only on relatively mature plants (Budge, Personal communication). *Pb* conidia have been observed in basil seed samples (Falach-Block *et al.,* 2019; Wood, 2021, personal communication) however, it is unlikely the propagules would remain viable for extended periods under unfavorable environmental conditions. Therefore, it is postulated that disease is propagated through mycelial infection inside the seed-coat (Jennings *et al.,* 2017).

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

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

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

Materials and methods

Fungicidal control in IPM programmes:

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.

Fungicide selection

A range of industry stakeholders and the AHDB crop protection team were consulted to select appropriate fungicides to be tested in 2021 for each pathogen in this programme. Selected products are shown in Table 1.

Table 1. Fungicides selected for testing on key crops in 2021.

Crop	Product	Active Ingredient
Tomato		
	Revus	Mandipropamid 250g/l
	Ranman Top	Cyazofamid 160g/l
	Previcur Energy	Fosetyl-aluminium 310g/l plus Propamocarb hydrochloride 530g/l
	Paraat	Dimethomorph 500g/kg
	Pure (Merck)	Difenoconazole *(material used in trial work was pure Difenoconazole is formulated at 250g/l Carial Star mix with 250g/l Mandipropamid)*
Column Stocks		
	Revus	Mandipropamid 250g/l
	Paraat	Dimethomorph 500g/kg
	Penncozeb	Mancozeb 75% w/w
	Previcur Energy	Fosetyl-aluminium 310g/l plus Propamocarb hydrochloride 530g/l
	Percos	Ametoctradin 300g/l Plus Dimethomorph 225g/l
Lettuce		
	Cleancrop Celeb	Azoxystrobin 250g/l
	Paraat	Dimethomorph 500g/kg
	Revus	Mandipropamid 250g/l
Spinach		
	Cleancrop Celeb	Azoxystrobin 250g/l
	Paraat	Dimethomorph 500g/kg
	Revus	Mandipropamid 250g/l

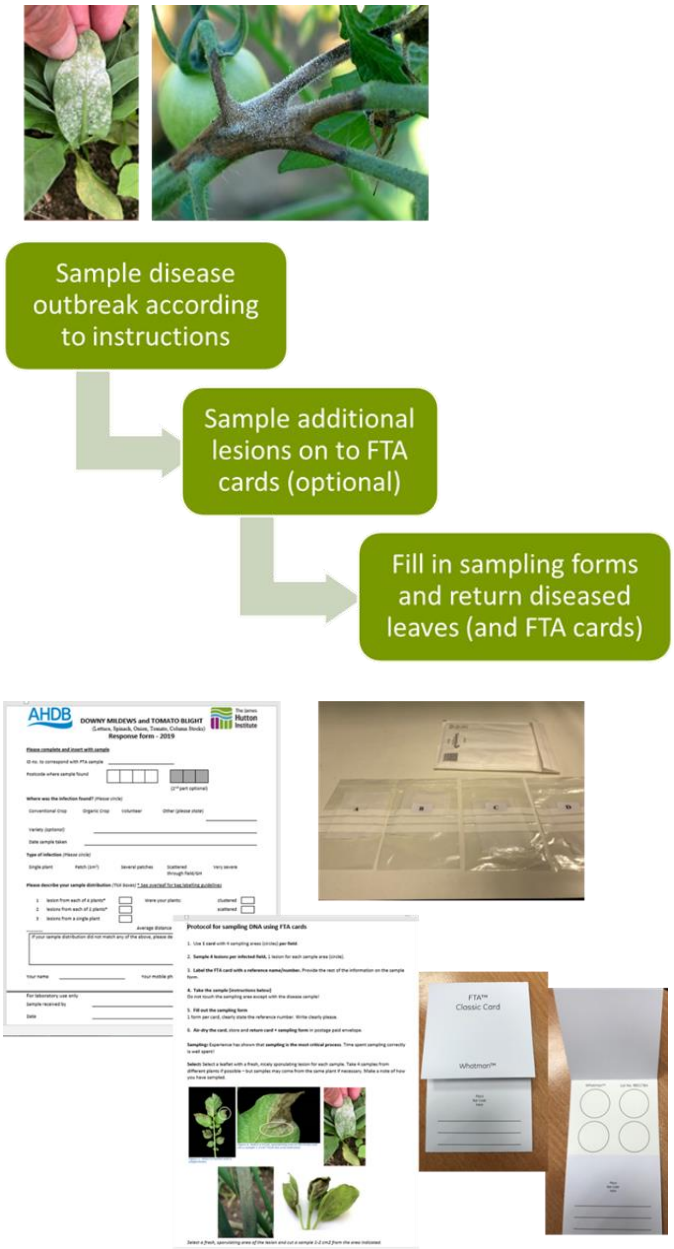

Figure 1. Sampling pack and instructions for oomycetes

Collection of isolates

Industry stakeholders were contacted through personal contacts, AHDB publications and newsletters and asked to submit disease samples for characterisation in the project. Fifty sample packs and instructions (Fig.1) were posted to stakeholders for postage-paid return, additional samples were also returned in unused packs sent in 2020. Growers (lettuce, spinach, column stocks, and tomato) were asked to sample 4 lesions per distinct disease outbreak wherever possible and to supply crop information. Additionally, an FTA® card was supplied onto which 4 different lesions could be sampled. This was to enable DNA from the outbreak to be stored (and analysed for genotypic characteristics) in addition to the live pathogen sample, or in cases where the live sample was not retrievable from the host material on receipt. The sampling strategy was aligned with that undertaken in the AHDB 'Fight Against Blight' campaign with a view to a longer-term system for outbreak alerts or pathogen characterisation being compatible.

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

Lettuce Markers:

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

Milestone 7.1) 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.

This milestone was previously achieved and work is currently underway to develop the SSR genotyping method for *B. lactucae* in an AHDB funded studentship held by Alicia Farmer. When samples of *B. lactucae* have been received for fungicide and race testing, DNA, spores and leaf material have also been stored and will be used in the marker development work. We have carried out race testing on the isolates obtained from 2021 outbreaks:

Race testing of *Bremia lactucae* isolates

B. lactucae isolates were assessed for race structure according to IBEB guidelines and protocols kindly supplied by Naktuinbouw in the Netherlands, who also supplied seed of the 16 current accessions in the official lettuce differential set (Set C). In brief, seed of each differential host (n=16) were germinated and grown on damp filter paper. Spores of each isolate maintained on lettuce seedlings were harvested into sterile distilled water. A sub-set of these spores were frozen for future use. Seedlings of each differential were inoculated with individual isolates of *B. lactucae* using a spore suspension containing 10 x 10^3 spores/ml. Each set of differentials was incubated in a large box with a lid to maintain high humidity, kept in a glasshouse at 15°C under natural daylight conditions. Disease was assessed 12 days post inoculation using the IBEB scale as described below (Fig. 2).

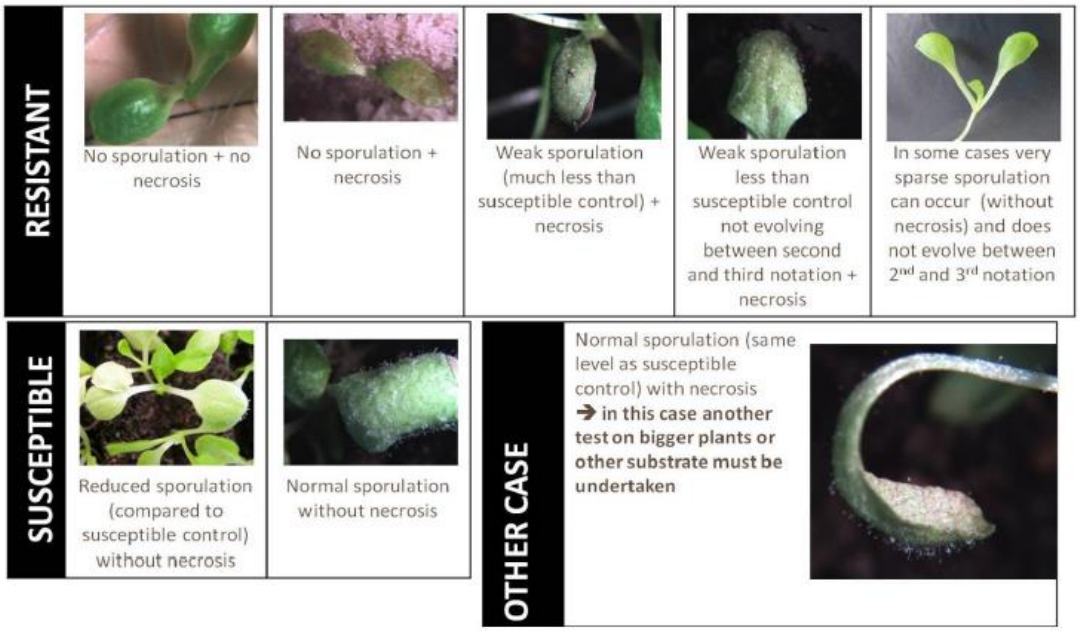

Figure 2. IBEB *Bremia* lactucae disease assessment scale.

Refining molecular diagnostic approaches to seed testing for viable downy mildew inoculum in sweet basil and spinach, reporting of a *Peronospora matthiolae* draft genome assembly and testing of column stock seed lots using qPCR:

Background

Early detection and diagnosis of pathogen on seed coats and within the seed is a highly effective strategy for reducing the incidence of serious disease outbreaks by preventing the sowing of infected material. Quantitative PCR (qPCR) has been demonstrated to be a highly effective technique for detecting the presence of pathogen DNA in a wide range of different horticultural crops, including basil and spinach (Shao & Tian, 2018; Feng *et al.,* 2014), and has been utilized previously for diagnosing seed-borne contamination in both crops (AHDB CP184 reports 2019, 2020).

Unfortunately a major limitation of DNA-based testing is that templates from both live and dead cells are amplified, often leading to an over-estimation of pathogen in a sample and

preventing accurate prediction of the levels of potential risk posed. Over-estimation of the amount of pathogen DNA in basil and spinach seed is an issue for producers utilizing physical decontamination strategies due to the presence of DNA from dead or unviable cells, making it difficult to demonstrate treatment efficacy. Growers must therefore risk sowing potentially contaminated seed, whilst paying higher prices and potentially needing to increase sowing rates to account for reduced levels of germination post-treatment.

Improving detection and quantification of live pathogen cells could therefore have positive implications for effectively validating seed decontamination strategies. Developing improved strategies detecting the amount of live pathogen propagules could help to quantify the risk posed by sowing infected seed lots for growers.

Viability qPCR, utilizing the photo-reactive dye Propidium Monoazide (PMA) has previously been utilized as a method to distinguish between the amount of live and dead *Pb* sporangia in spore suspensions collected from infected plant material (1 x 10^6/ml; CP184 Annual report 2020). However, the results were inconclusive, due in part to a reduction in the DNA content in dead cell samples compared to live ones on heating at 95°C. Here we report on the optimization of the viability PCR technique for determining the amount of live *P. belbahrii* and *P. effusa* sporangia isolated from infected plant material.

Whilst standard and viability PCR approaches can be implemented where assays have been designed and thoroughly validated, detection of new or novel downy mildews requires design and testing for specificity, which can be challenging and time-consuming process. A number of important ornamental species are affected by downy mildew leading to serious economic penalties, including Agastache, Coleus (*Peronospora belbahrii),* Column stock *(Peronospora matthiolae),* Hebe *(Peronospora grisea),* Impatiens (*Plasmopara obducens)* and Poppy (*Peronospora somniferum).* Testing of seed for pathogens, including downy mildew, can be utilized as an effective disease prevention strategy to limit negative impacts in commercial ornamental species. Access to next-generation sequencing provides enhanced knowledge of pathogen genomes and enables specific diagnostic assays to be designed to detect important agents. We also report the construction of a draft column-stock downy mildew (*P. matthiollae)* genome, and subsequent phylogenetic analysis of related downy mildew species and the testing of a new qPCR assay for detecting this pathogen (*P. matthiollae*) in seed.

Quantification of spore suspensions

Sporangial suspensions of *P. belbahrii, P. effusa* and *P. matthiolae* (1 x 10^6/ml) for extracting DNA for generating the qPCR control standards were quantified in triplicate using a haemocytometer visualised on an Olympus BN2 microscope at 20 x magnification.

DNA extraction

DNA extraction was performed using a DNeasy Plant mini kit (Qiagen, UK) according to the manufacturer's protocol; sporangial suspensions 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.

Quantitative PCR

Extracted DNA was analysed on an ABI Step one plus in a 96-well format using 10 µl volumes with triplicate technical replicates per sample.

Assays for detecting *P. belbahrii, P. effusa* and *P. matthiolae* (*P. belbahrii:* PbITS2-F: 5'-CTGAACAGGCGCTGATTG, PbITS2-R: GCAACAGCAAAGCCAATTC (Shao & Tian, 2018); *P. effusa:* PfsITSF: GTTCGATTCGCGGTATGATT, PfsITSR: TCACACAGCAAAGCCAATTC (Feng *et al.,* 2014); *P. matthiolae:* PmITS.F: TGGCTTCGGCTGAACTG; PmITS.R: CCAAGTCACACACGCTAC) were validated by screening pure DNA extracted from spores over a 100 ng-10 fg/µl dilution series. The temperature cycle for PCRs consisted of an initial denaturation (96°C) for 2 mins, followed by 40 amplification cycles alternating between 96°C for 15 sec and 60°C for 30 sec extension time per cycle. The number of cycles after which DNA could be detected against the baseline is termed the cycle threshold (Ct). The Ct is the number of cycles of PCR for the fluorescence signal generated during amplification to exceed a threshold. The greater the number of amplification cycles required to detect DNA, the lower the starting quantity in the sample.

DNA extraction from *Matthiola incana* seed

3 x 50 seed samples of seed lots CP 12 - 17 (table 3) were collected in individual 2.5 ml micro-centrifuge tubes containing 2 x 3 mm steel balls. Seed samples were ground in liquid nitrogen for 60 secs at 1000 rpm using a Genogrinder (Spex Sample Prep, USA). DNA extractions on *P. matthiolae* seed were performed using a Wizard Food kit (Promega, UK) was used to extract DNA according to the manufacturer's protocol.

***Peronospora matthiolae* genome sequencing and assembly**

Sporangia were collected from sporulating *M. incana* plants and ground in 100 µl of extraction buffer with a small quantity silver sand. DNA extraction was performed using a DNeasy Plant mini kit (Qiagen, UK) according to the manufacturer's protocol. DNA was checked for quality and concentration using a nanodrop spectrophotometer and Qubit analyser and diluted to 100 ng/µl (2 µg total). DNA was sequenced on the Oxford Nanopore Technologies MinION using a R 9.4.1 flowcell and sequencing kit SQK-LSK 109, Long reads were assembled using Flye 2.8.3 and polished with Medaka 0.11.5. The assembly was filtered for contaminant contigs using blastn and the nt-database. Throughout the process, assembly quality and gene content were monitored using BUSCO v. 4.0.5 and Quast v. 5.0.2. Before annotation repeats were modelled and soft-masked using RepModeler v. 2.0.1 and RepMasker v. 4.1.0 respectively. The assembly was annotated in two runs with BRAKER 2.1.5 using the OrthoDB database of eukaryotic proteins v. 10 and aligned RNA short reads as references and combining the results. The *P. matthiolae* genome assembly is deposited at Ensembl Protists as GCA_921007115.1 within project PRJEB48439 and will be made publicly available upon publication of the accompanying manuscript (in preparation).

Phylogenetic analysis of *P. matthiolae* and closely related downy mildew spp.

Genetic distances based on housekeeping gene sequences (Beta-tublin) were estimated using the Adegenet package in R and neighbour joining trees were constructed to enable relative distances to be visualised. Sequences from *Hyaloperonospora arabidopsidis, H. brassicae, H. parasitica, Peronospora belbahrii, P. farinosa (*syn. *P. schactii)* and *P. effusa* were included in the analysis.

PMA-PCR on oomycete spore-suspensions

PMA PCR kits and PMA-lite illuminator were purchased from Biotium (VWR, U.K.). *P. belbahrii* and *P. effusa* spores (1 x 10^6/ml, respectively) were prepared by washing the leaves of freshly sporulating plants with distilled water. 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. Downy mildew spores in one sub-sample were inactivated by heating at 95°C for 5 minutes. PMA treatment and qPCR were conducted according to the manufacturer's protocol (https://biotium.com/technology/pma-for-viability-pcr/), with 15 minutes LED illumination. DNA was extracted from spore suspensions using a Plant DNeasy mini kit (Qiagen, UK) and standard dilution series (100 ng/µl – 10 fg/µl DNA/µl) was prepared from purified *P. belbahrii* and *P. effusa* DNA, respectively. A dilution series was also prepared from DNA extracted from the respective cell cultures to test the limit of detection of the assay. Pure and diluted DNA extracted from live/dead and PMA-treated live/dead samples were analyzed 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 dead 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 been obtained the change in dCt can be calculated to provide a quantitative estimation of the quantity 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:

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

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

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

Results

Fungicidal control in IPM programmes:

Column Stocks (*Peronospora matthiolae*)

Samples were received from 7 individual disease outbreaks (Pp1-7) in 2021; these were returned as bulk column stock leaf samples. Six isolates of *Peronospora matthiolae* were obtained from the 7 bulk leaf samples (Table 2). Isolates were transferred to fresh, disease-free leaves of *Matthiola indica* and were maintained for inoculum production by regular sub-culturing onto new plants before being tested for fungicide insensitivity.

Table 2. Isolates of *Peronospora matthiolae* obtained from 2021 outbreaks

Isolate ID code	County	Date Collected	Date Received
2021_Pp1	Norfolk	15/05/2021	17/05/2021
2021_Pp3	Cambridgeshire	19/06/2021	22/06/2021
2021_Pp4	Cambridgeshire	19/06/2021	22/06/2021
2021_Pp5	Newtonards	18/06/2021	22/06/2021
2021_Pp6	Cornwall	04/07/2021	07/07/2021
2021_Pp7	Co. Armagh	04/07/2021	08/07/2021

Six individual isolates were tested with 5 fungicides (Table 3) and 5 replicate plants were tested for each isolate/fungicide combination. Inoculation was carried out 1 day after fungicide application. Plants were assessed for disease incidence 14 days after inoculation by counting 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 3. Fungicide product and application rate information.

Product	Formulation	Water Volume Max (L)	Dose Rate	Concentration	Tank Mix (ppm)
Revus	250g/l Mandipropamid	1000	0.6L/Ha	0.6ml/L	150
Paraat	500g/kg Dimethomorph	1000	3kg/Ha	3g/L	1500
Previcur Energy	310g/l Fosetyl-aluminium + 530g/l Propamocarb hydrochloride	1000	2.5L/Ha	2.5ml/L	775
Percos	300g/l Ametoctradin + 225g/l Dimethomorph	500	3.2L/Ha	6.4ml/L	1920 1440
Penncozeb	75% w/w Mancozeb	200	1.7kg/Ha	8.5ml/L	6375

Good levels of disease (60-76% incidence) were observed in the untreated control with all isolates tested (Fig 3). Levels of disease control ranged from 52-82% across all isolates tested after treatment with Revus (Mandipropamid) compared with the untreated controls (Fig 4). Results show less effective control overall than 2019 data (range 80-86% control). Variable levels of control (28-68%) between isolates were observed with Previcur Energy (Fosetyl-Aluminium/Propamocarb hydrochloride) (Fig 5). This was in line with the range noted in 2019 (31-67%). It may be worth cross referencing the source of the more insensitive isolates across both years.

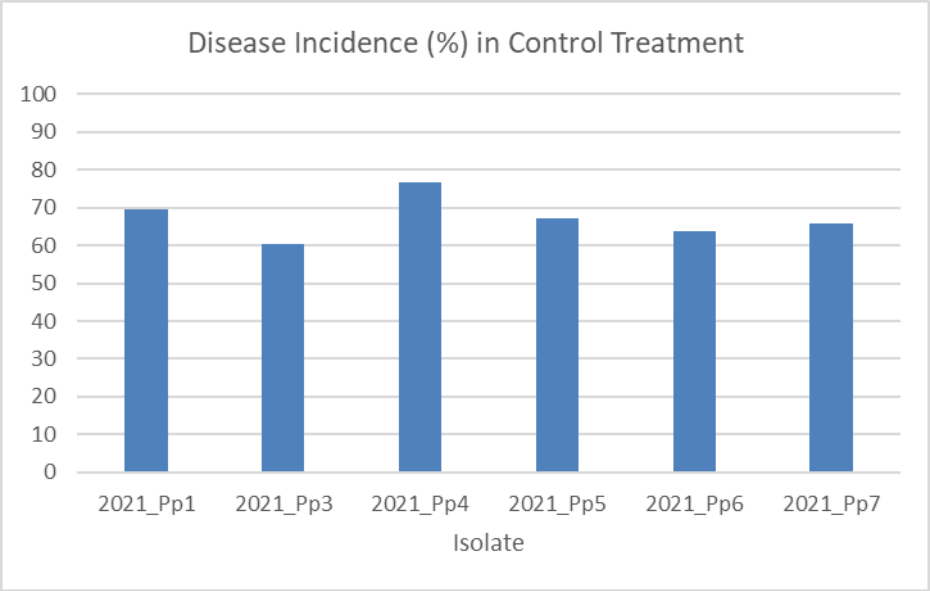

Figure 3. Disease Incidence caused by each isolate of *P. matthiolae* in the untreated control.

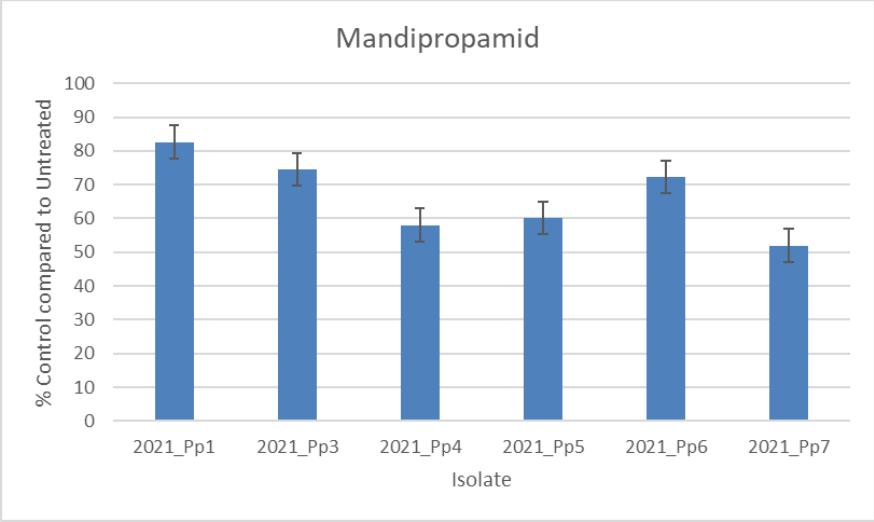

Figure 4. Mean percentage disease control (as % of untreated control) of each isolate of *P. matthiolae* observed following application of Mandipropamid.

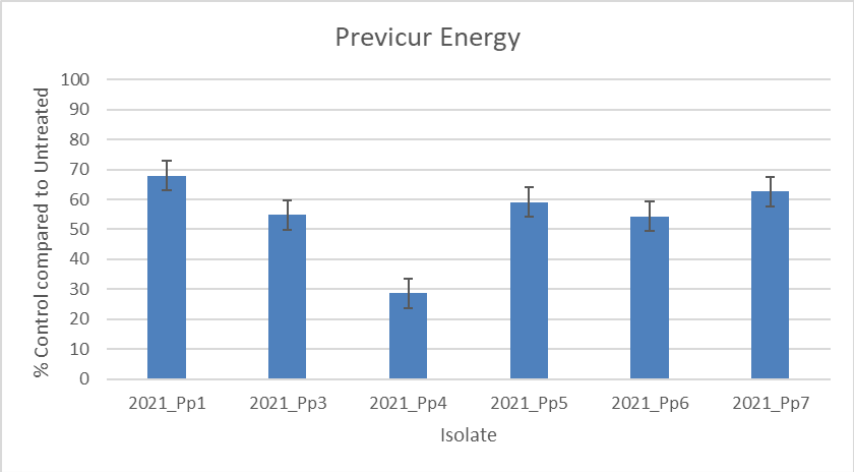

Figure 5. Mean percentage disease control (as % of untreated control) of each isolate of *P. matthiolae* observed following application of Previcur Energy.

Variable but low levels of control (10-50%) of all isolates was achieved with Paraat (Dimethomorph) (Fig 6). This is in contrast with 2019, where 80-90% control was observed in all isolates apart from 2019_Pp4 (40%) suggesting that either insensitivity is now more widespread or that there is a link (source) between the 2019_Pp4 sample and the range of isolates obtained in 2021.

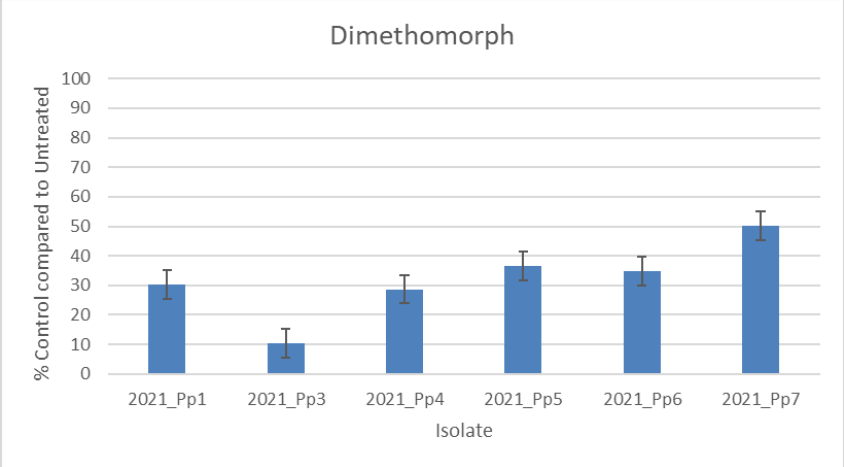

Figure 6. Mean percentage disease control (as % of untreated control) of each isolate of *P. matthiolae* observed following application of Dimethomorph.

Moderate and variable disease control (34-66%) was shown with application of Percos (Amectotradin/Dimethomorph) across the isolates tested in 2021 (Fig 7). This product was not previously tested in 2019. Control is slightly improved on that seen with dimethomorph alone in 2021. The concentration of dimethomorph in Percos is lower than in dimethomorph as a single product and it is difficult therefore to determine the effect of Ametoctradin. Moderate and variable disease control (15-65%) was shown after treatment with Mancozeb across the isolates tested in 2021 (Fig 8). This product was not previously tested in 2019.

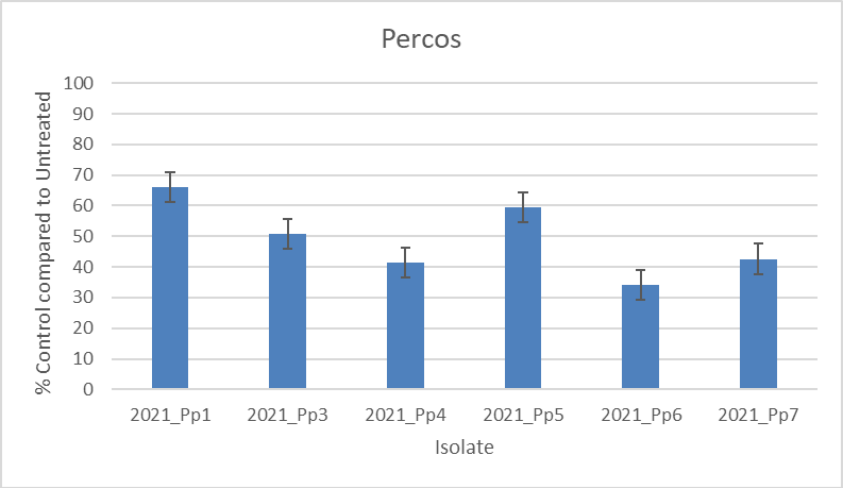

Figure 7. Mean percentage disease control (as % of untreated control) of each isolate of *P. matthiolae* observed following application of Percos.

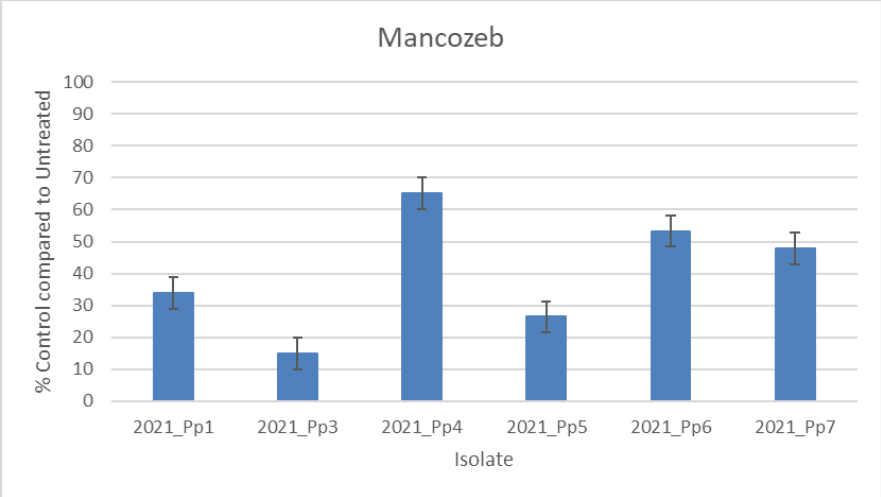

Figure 8. Mean percentage disease control (as % of untreated control) of each isolate of *P. matthiolae* observed following application of Mancozeb.

Spinach (*Peronospora effusa*)

Samples from 7 individual downy mildew disease outbreaks in spinach (Pe1-7) were received in 2021. From these outbreaks 10 individual isolates were obtained (Table 4), maintained on leaves of the susceptible variety Viroflay and assessed for fungicide sensitivity and race profile.

Ten isolates of *Peronospora effusa* were each tested for sensitivity to three fungicides at 4 concentrations as listed in Table 5. Fungicide concentrations were chosen based on existing information on use, or baseline sensitivity testing of the individual products. Spinach plants (cv. Viroflay) 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 before inoculation. Inoculation was carried out using a sporangial suspension of *P. effusa*. Inoculated plants were incubated for 11 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 at different levels of

fungicide. 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 4. Isolates of *Peronospora effusa* obtained from 2021 outbreaks.

Isolate ID code	County	Outbreak size	Date Collected	Date Received
2021_Pe2A	Kent	Scattered	12/08/2021	16/08/2021
2021_Pe2B	Kent	Scattered	12/08/2021	16/08/2021
2021_Pe2C	Kent	Scattered	12/08/2021	16/08/2021
2021_Pe2D	Kent	Scattered	12/08/2021	16/08/2021
2021_Pe3A	Lincolnshire	Patch (1m2)	16/08/2021	19/08/2021
2021_Pe3B	Lincolnshire	Patch (1m2)	16/08/2021	19/08/2021
2021_Pe3C	Lincolnshire	Patch (1m2)	16/08/2021	19/08/2021
2021_Pe3D	Lincolnshire	Patch (1m2)	16/08/2021	19/08/2021
2021_Pe5A	Shropshire	Scattered	17/09/2021	23/09/2021
2021_Pe6A	Shropshire	Scattered	17/09/2021	23/09/2021

Table 5. Information relating to fungicides and application rates tested on *P. effusa* isolates from spinach.

Active ingredient	Mandipropamid	Dimethomorph	Azoxystrobin
Product	Revus	Paraat	Cleancrop Celeb
Formulation	250g/l	500g/kg	250g/l
Max water volume (L)	1000	1000	300
Min water volume (L)	200	600	150
Dose rate	0.6L/Ha	3kg/Ha	1.0L/Ha
Max tank mix (ppm)	750	2500	1666
Min tank mix (ppm)	150	1500	833
Range of concentrations tested for EC50 calculation (ppm)	0, 10, 100, 750	0, 10, 100, 900	0, 100, 1000, 1666

Moderately high levels of disease (43-60% of diseased leaf area) were observed in the untreated control plants, indicating that the test method and the inoculum quality of all the tested isolates was satisfactory (Fig.9). Dose responses for Mandipropamid, Dimethomorph and Azoxystrobin were appropriate for the calculation of EC50 values at the range of concentrations tested. Data is presented as percentage disease control of each isolate achieved with application of Mandipropamid (Fig 10), Dimethomorph (Fig. 11) and Azoxystrobin (Fig. 12). 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 spinach downy mildew disease severity compared with the untreated control was calculated for each individual active ingredient. Azoxystrobin demonstrated consistently high levels of disease control at field rate (99-100%) across all

isolates tested (Fig. 12). Mandipropamid (100%) and dimethomorph (100%) showed complete control of spinach downy mildew at the highest rates tested (Fig 10 and Fig 11 respectively).

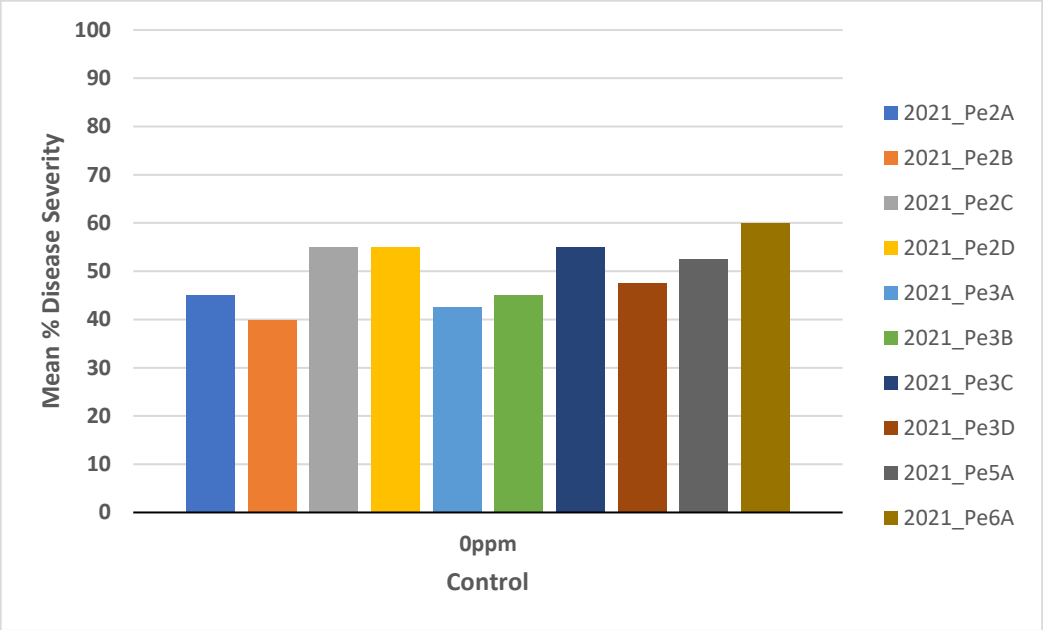

Figure 9. Mean disease severity (percentage infected leaf area) for each isolate of *Peronospora effusa* tested in the untreated control.

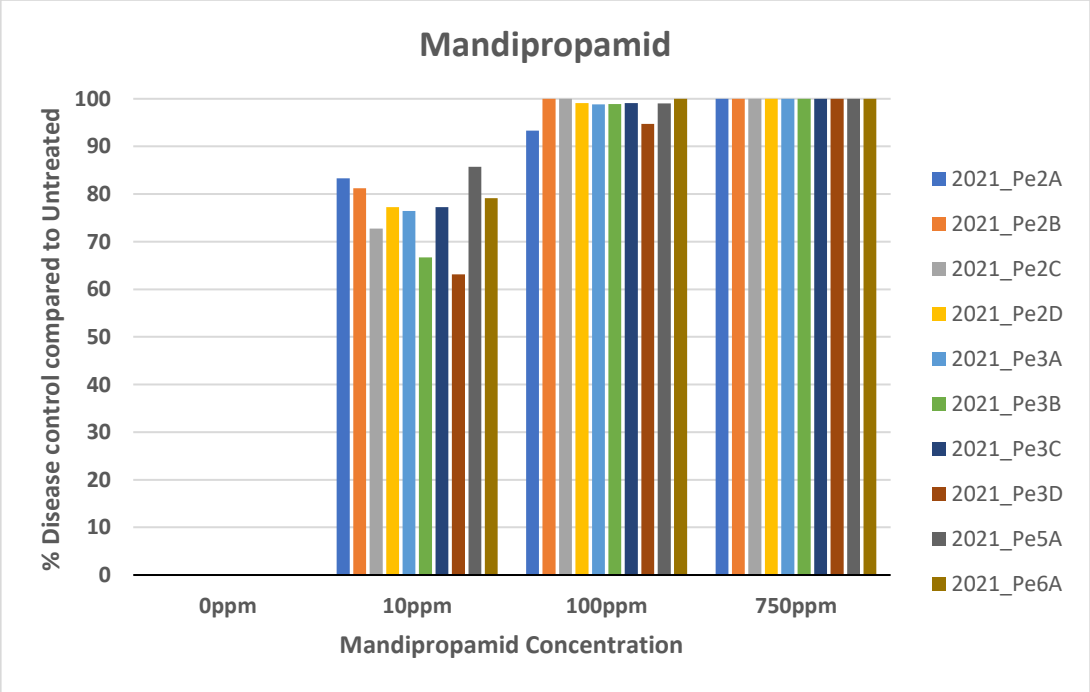

Figure 10 Mean percentage disease control of 10 isolates of *P. effusa* by mandipropamid applied at a range of concentrations compared with an untreated control.

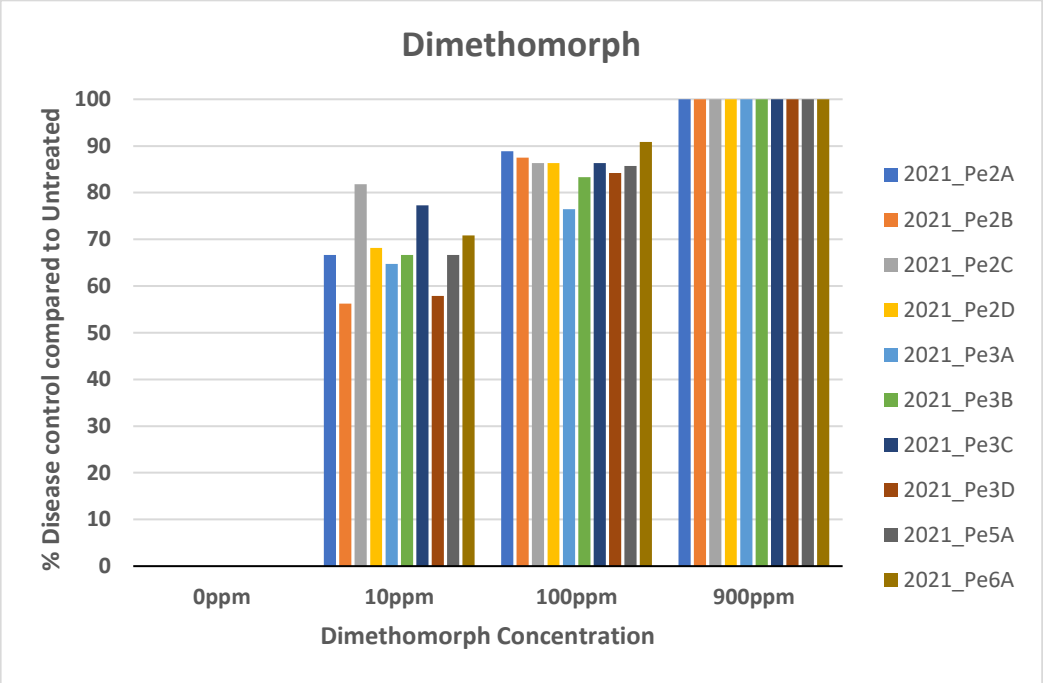

Figure 11. Mean percentage disease control of 10 isolates of *P. effusa* by Dimethomorph applied at a range of concentrations compared with an untreated control.

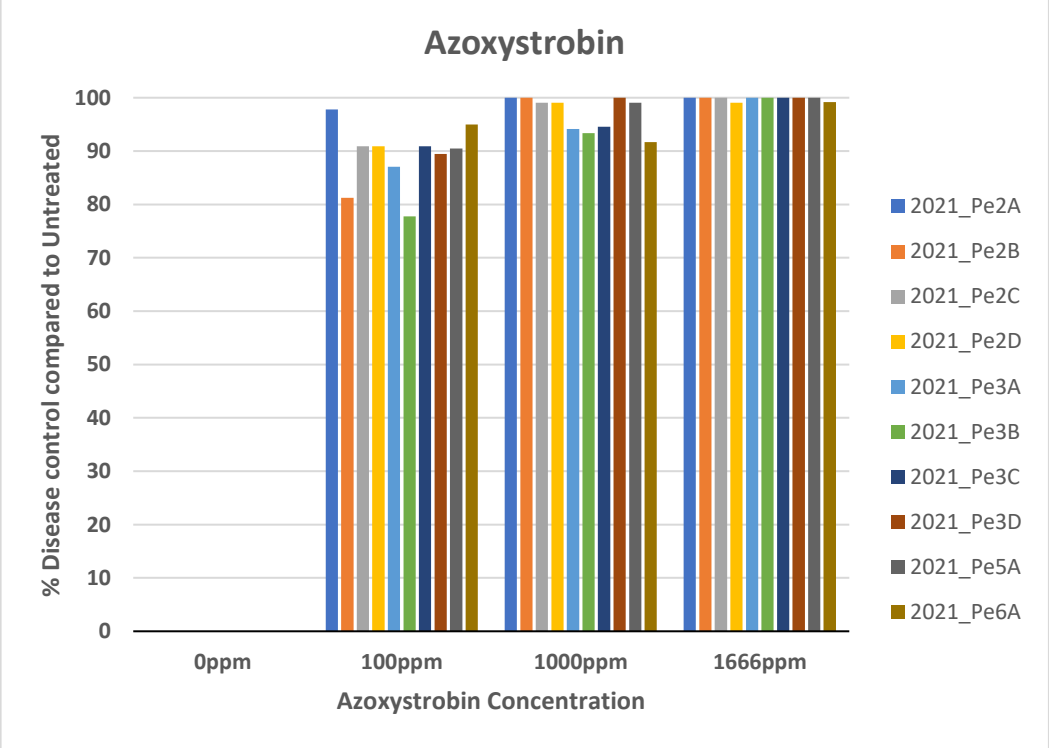

Figure 12. Mean percentage disease control of 10 isolates of *P. effusa* by Azoxystrobin applied at a range of concentrations compared with an untreated control.

Estimated EC50 values (mean, minimum and maximum) for each fungicide tested are given in Table 6. There were differences observed in EC50 value between isolates across all 3 products tested but these differences were not shown to be statistically significant.

Table 6. Mean, minimum and maximum EC50 values for Mandipropamid, Dimethomorph and Azoxystrobin calculated from data obtained from 10 isolates of *P. effusa* tested at a range of concentrations**.**

Fungicide	Mandipropamid	Dimethomorph	Azoxystrobin
Number of isolates	10	10	10
Mean EC_{50} (mg/L)	**2.29**	**3.86**	**5.14**
EC_{50} min (mg/L)	1.35	1.82	3.31
EC_{50} max (mg/L)	4.04	18.13	9.95

Lettuce (*Bremia Lactucae*)

Samples were received from 14 individual disease outbreaks (Bl1-14) in 2021. From each of these 14 outbreaks FTA samples were obtained from 4 individual lesions in most cases. It was not possible to obtain viable isolates from all samples received, either due to the condition of the sample or poor sporulation of subsequent leaf cultures. Twenty-one isolates from 7 individual outbreaks (Table 7) were successfully isolated from disease samples and maintained on seedlings of variety Green Towers. These isolates were assessed for fungicide sensitivity and race profile. Spore suspensions and inoculated seedlings were stored at -20°C and -80°C for future use.

Twelve isolates of *B. lactucae* were tested for sensitivity to 3 fungicides at 4 concentrations (Table 8). The 12 isolates included 3 obtained from outbreaks in 2020, two control isolates supplied by Naktuinbouw in the Netherlands, and 7 selected across different outbreaks in 2021 (Table 9). Fungicide concentrations were chosen based on existing information or baseline sensitivity testing of 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 24h before inoculation. Inoculation was carried out using a sporangial suspension of *B. lactucae* obtained from diseased leaves according to the IBEB protocol. Inoculated plants were incubated under glasshouse conditions for 11 days before 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 at different levels of fungicide. 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 7. Isolates of *Bremia lactucae* obtained from 2021 lettuce downy mildew outbreaks. Isolates with the same number and different letters originate from different samples within the same outbreak.

Isolate ID code	County	Outbreak size	Date Collected	Date Received
2021_Bl1A	Cambridgeshire	Patch (1m)	07/07/2021	09/07/2021
2021_Bl1B	Cambridgeshire	Patch (1m)	07/07/2021	09/07/2021
2021_Bl1J	Cambridgeshire	Patch (1m)	07/07/2021	09/07/2021
2021_Bl1L	Cambridgeshire	Patch (1m)	07/07/2021	09/07/2021
2021_Bl2B	Cambridgeshire	Several patches	12/08/2021	16/08/2021
2021_Bl2D	Cambridgeshire	Several patches	12/08/2021	16/08/2021
2021_Bl2H	Cambridgeshire	Several patches	12/08/2021	16/08/2021
2021_Bl3A	Fife	Large >50m^2	17/08/2021	17/08/2021
2021_Bl3B	Fife	Large >50m^2	17/08/2021	17/08/2021
2021_Bl3C	Fife	Large >50m^2	17/08/2021	17/08/2021
2021_Bl3D	Fife	Large >50m^2	17/08/2021	17/08/2021
2021_Bl4C	Cambridgeshire	Scattered	17/08/2021	19/08/2021
2021_B8A	Lincolnshire	Scattered	07/09/2021	09/09/2021
2021_B8B	Lincolnshire	Scattered	07/09/2021	09/09/2021
2021_B8C	Lincolnshire	Scattered	07/09/2021	09/09/2021
2021_B8D	Lincolnshire	Scattered	07/09/2021	09/09/2021
2021_Bl9	Dundee	Scattered	22/09/2021	22/09/2021
2021_B11A	Norfolk	Very Severe	05/10/2021	12/10/2021
2021_B11B	Norfolk	Very Severe	05/10/2021	12/10/2021
2021_B11C	Norfolk	Very Severe	05/10/2021	12/10/2021
2021_B11D	Norfolk	Very Severe	05/10/2021	12/10/2021

Table 8. 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**

Table 9. Isolates of *B. lactucae* included in fungicide sensitivity testing in 2021 (grouped into 2020 isolates, 2021 isolates and Naktuinbouw.reference isolates from the Netherlands)

Isolate ID code	County	Outbreak size	Date Collected	Date Received
2020_Bl2	West Sussex	Patch (1m2)	25/08/2020	28/08/2020
2020_Bl4E	Cambridgeshire	Several patches	16/09/2020	18/09/2020
2020_Bl4G	Cambridgeshire	Several patches	16/09/2020	18/09/2020
2021_Bl1A	Cambridgeshire	Patch (1m)	07/07/2021	09/07/2021
2021_Bl1J	Cambridgeshire	Patch (1m)	07/07/2021	09/07/2021
2021_Bl2B	Cambridgeshire	Several patches	12/08/2021	16/08/2021
2021_Bl3A	Fife	Large >50m^2	17/08/2021	17/08/2021
2021_Bl3C	Fife	Large >50m^2	17/08/2021	17/08/2021
2021_B8A	Lincolnshire	Scattered	07/09/2021	09/09/2021
2021_Bl9C	Dundee	Scattered	22/09/2021	22/09/2021
NAK_EU16				
NAK_EU36				

Moderately high levels of disease (35-72.5% 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 (Fig.13). Dose responses for Mandipropamid, Dimethomorph, and Azoxystrobin were appropriate for the calculation of EC50 values at the range of concentrations tested. Data is presented as percentage disease control of each isolate achieved by application of Mandipropamid (Fig 14), Dimethomorph (Fig. 15) and Azoxystrobin (Fig. 16). 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 (99-100%) across all isolates tested (Fig. 14). Similarly, Dimethomorph (95-100%) and Azoxystrobin (90-100%) showed good control of lettuce downy mildew at the highest rates tested (Fig 15 and Fig 16 respectively).

Estimated EC50 values (mean, minimum and maximum) for each fungicide tested in 2019 and 2021 are given in Table 10. Some large differences in the min and max values were noted for some isolates, particularly for Dimethomorph and Azoxystrobin. A high mean EC50 and max EC50 value was observed for Azoxystrobin in 2021, this is a result of a single isolate (2021_BI9C) causing more symptoms on the control plants and those treated with 100ppm azoxystrobin. No conclusions should be drawn from the data as it may just represent variation and be irrelevant to in-field control.

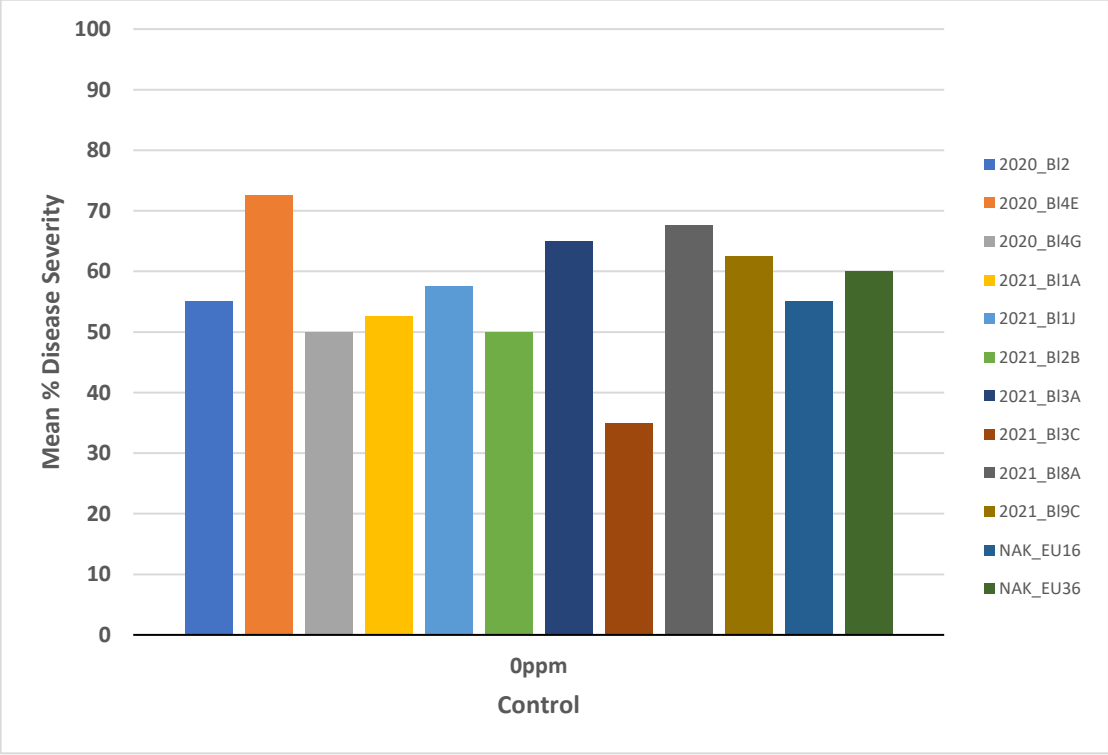

Figure 13. Average disease severity (percentage infected leaf area) for each isolate of *B. lactucae* tested in the untreated control.

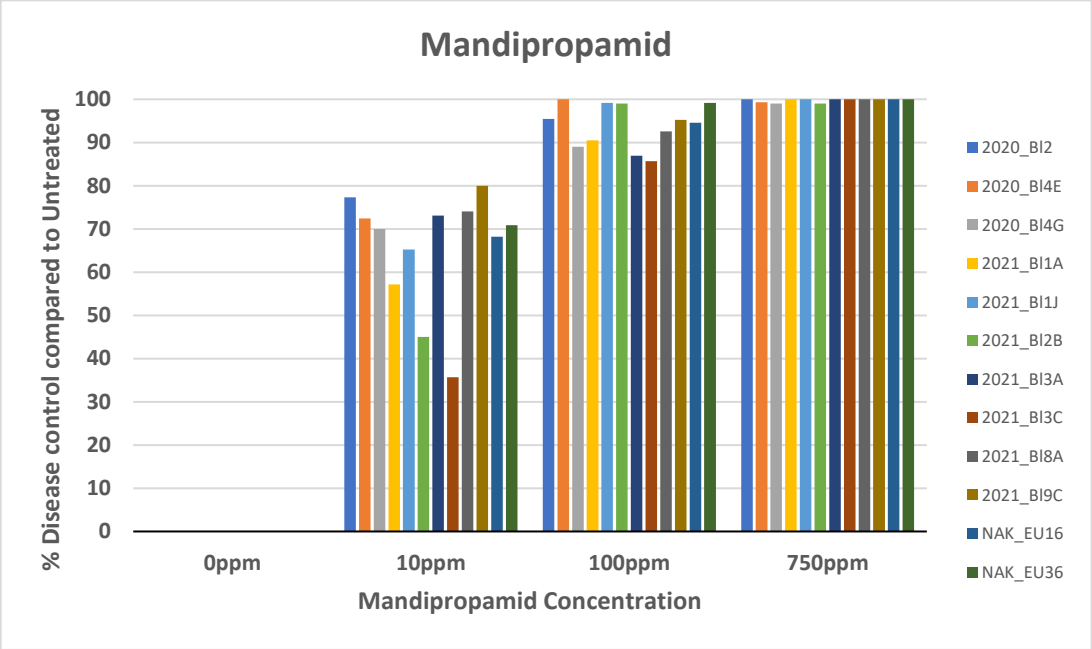

Figure 14. Mean percentage disease control of 10 isolates of *B. lactucae* by mandipropamid applied at a range of concentrations compared with an untreated control.

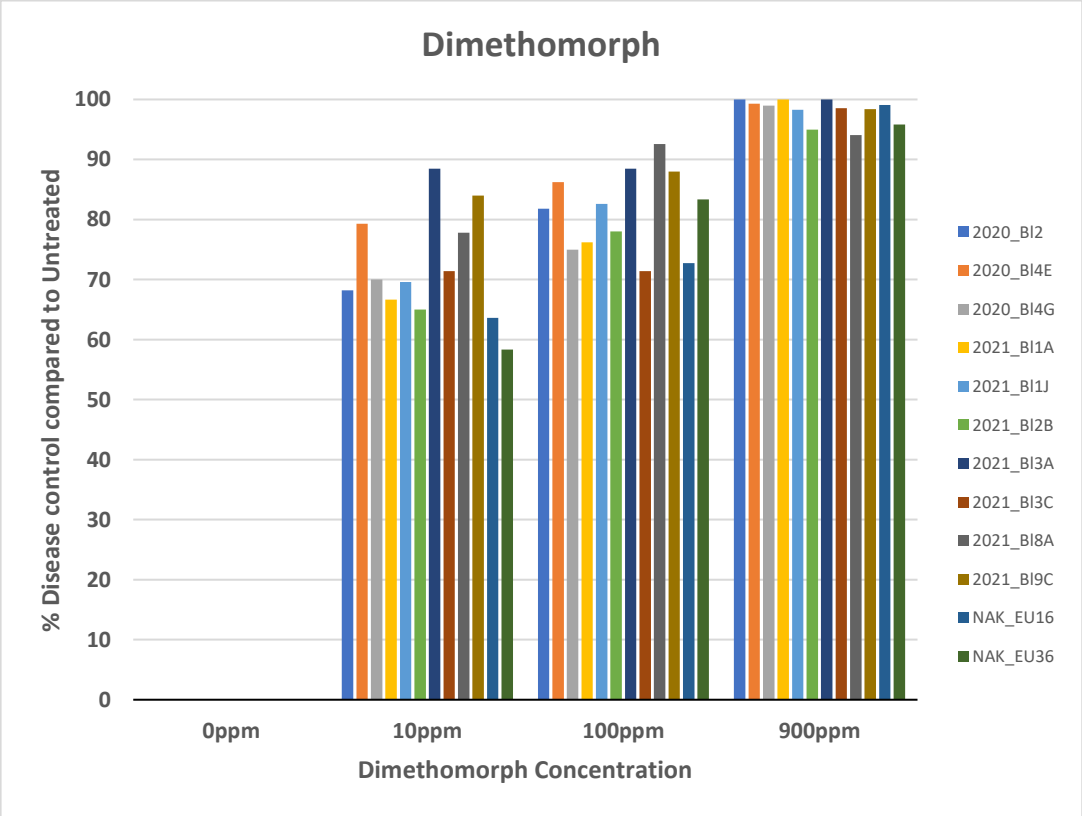

Figure 15. Mean percentage disease control of 10 isolates of *B. lactucae* by Dimethomorph applied at a range of concentrations compared with an untreated control.

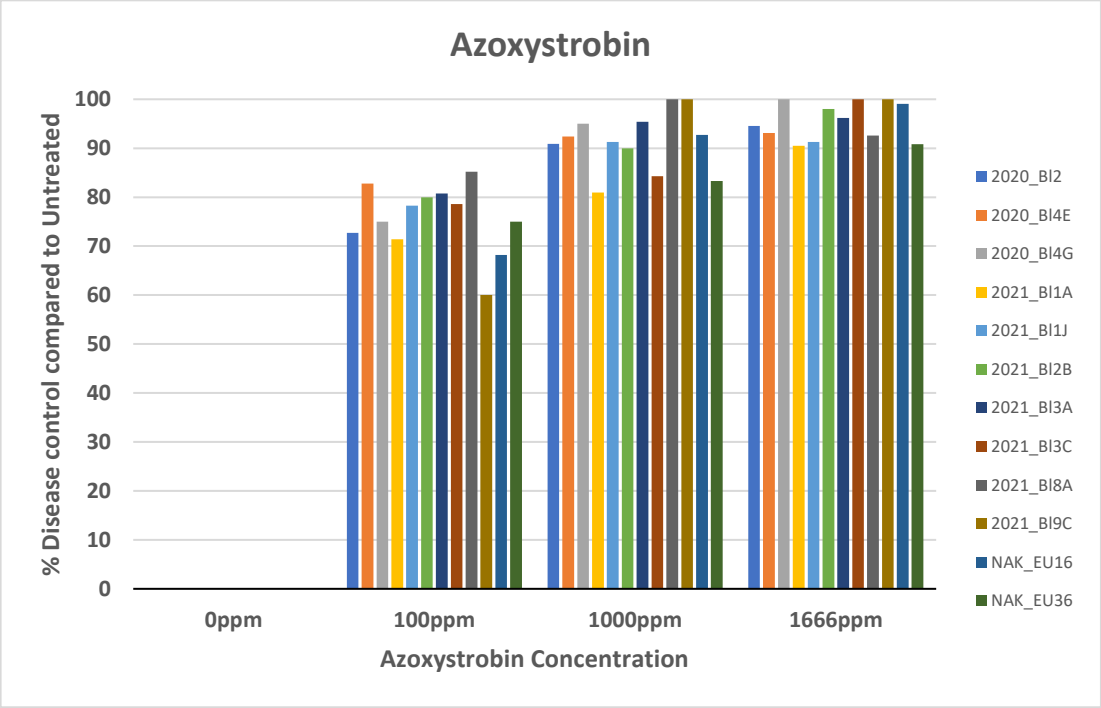

Figure 16. Mean percentage disease control of 10 isolates of *B. lactucae* by Azoxystrobin applied at a range of concentrations compared with an untreated control.

Table 10. Mean, minimum and maximum EC50 values for Mandipropamid, Dimethomorph and Azoxystrobin calculated from data obtained from isolates of *B. lactucae* in 2019 and 2021.

Fungicide	Mandipropamid		Dimethomorph		Azoxystrobin	
Year	2019	2021	2019	2021	2019	2021
Number of isolates	15	12	15	12	15	12
Mean EC_{50} (mg/L)	**15.6**	**5.3**	**38**	**3.7**	**233**	**15.2**
EC_{50} min (mg/L)	3.2	1.35	3	1.64	8	6.03
EC_{50} max (mg/L)	42.9	33.02	125	9.95	820	99.22

Lettuce Markers

Results of *B. lactucae* race tests

Results of race testing of 21 isolates of *B. lactucae* are presented in Table 11. Two races could be identified as a race previously designated by the IBEB committee (BL:35EU).

There were no apparent problems with the tests; all the universally susceptible control seedlings (cv. Green Towers) became infected with each isolate, providing confidence in

the test method. Following the completion of this project, further analysis of this race data in comparison with all the publicly available data in the Bremia database will be continued to draw ongoing comparisons where possible. Race testing results for all of the isolates tested from 2019 to 2021 were included in the IBEB EU Groslist 2021.

Table 11. Race test results of 21 isolates obtained from 2021 outbreaks.

	Green Towers	Dandie	R4T57 D	UC DM14	NunDm15	CG Dm16	Colorado	FrRsal-1	Argeles	RYZ 2164	RYZ 910457	Bedford	Balesta	Bartoli	Design	Kibrille	C Sextet Code
		Dm3	Dm4	Dm14	Dm15	Dm16	Dm18	Rsal-1	R38								
Grid Position		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	
Sextet Value		1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	
2021_Bl1A	+	-	+	+	+	+	+	-	+	-	-	-	+	-	-	+	62-34-04
2021_Bl1B	+	-	+	+	-	+	+	-	+	-	-	-	+	-	-	-	54-34-00
2021_Bl1J	+	+	+	+	-	+	+	-	+	-	-	-	+	-	-	-	55-34-00
2021_Bl1L	+	-	+	+	-	+	+	-	+	-	-	+	+	-	-	+	54-50-04
2021_Bl2B	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+	55-38-07
2021_Bl2D	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	54-38-07
2021_Bl2H	+	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-	54-34-01
2021_Bl3A	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	+	62-07-04
2021_Bl3B	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	62-06-00
2021_Bl3C	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	30-09-00
2021_Bl3D	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	30-09-00
2021_Bl4C	+	+	+	+	-	+	+	-	+	-	+	-	-	-	-	+	55-10-04
2021_B8A	+	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	62-38-06
2021_B8B	+	-	+	+	-	+	-	-	+	+	+	-	+	-	-	+	22-46-04
2021_B8C	+	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	62-38-06
2021_B8D	+	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	62-38-06
2021_BL9	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	-	62-07-03
BL:35EU	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06
2021_BL11A	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06
2021_BL11C	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	62-15-06
2021_BL11B	+	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+	54-07-06
2021_BL11D	+	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+	54-07-06

Refining molecular diagnostic approaches to seed testing for viable downy mildew inoculum in sweet basil and spinach, reporting of a *Peronospora matthiolae* draft genome assembly and testing of column stock seed lots using qPCR:

Optimizing PMA-qPCR approaches for detecting viable downy mildew inoculum from sweet basil and spinach

The amount of DNA in viable sweet basil and spinach downy mildew sporangia was quantified in live untreated and dead, heat-treated samples using PMA-qPCR.

Heat-treatment of *P. belbahrii* sporangia (1 x10^6/ml) resulted in a significant increase of Ct 4.04 in the dead compared to live, untreated samples (Table 12), using the PMA-qPCR approach. The increase in Ct indicated more than a 10x reduction in *P. belbahrii* DNA in the heat-treated samples, compared to the live, untreated samples. This indicated that the heat treatment at 95°C for 5 min effectively disrupted cell walls of the sporangia, enabling the PMA to bind to the (pathogen) DNA in the heat-treated samples and inhibit PCR amplification of these templates. An average difference in Ct of 4.19 was observed between samples comprising the serial dilutions prepared from DNA extracted from the 1 x 10^6/ml spore suspension.

Table 12. Cycle threshold (Ct) values for DNA extracted from live and dead *P. belbahrii* spores treated (+) or untreated (-) with PMA dye (1x 10^6/ml), and serial dilution of samples mimicking spore suspensions 1 x 10^4 to 1 x 10^2/ml.

P. belbahrii spores (ml)	Sample	Ct Mean	Ct +/- PMA	Ct Live vs Dead
1 x 10^6	Alive 1-	8.84235		
	Alive 1+	11.7809	2.93859	
	Dead 1-	13.3074		
	Dead 1+	20.4394	7.13198	4.19
1 x10^5	Alive 2-	12.14		
	Alive 2+	15.13	2.99	
	Dead 2-	16.61		
	Dead 2+	23.64	7.03	4.04
1 x10^4	Alive 3+	15.33		
	Alive 3-	18.43	3.11	
	Dead 3-	19.89		
	Dead 3+	26.84	6.94	3.84
1 x10^3	Alive 4-	18.7		
	Alive 4+	21.78	3.08	
	Dead 4-	23.26		
	Dead 4+	30.25	6.99	3.91
1 x10^2	Alive 5-	22.34		
	Alive 5+	25.28	2.94	
	Dead 5-	26.67		
	Dead 5+	34.6	7.93	4.99

Heat treatment was observed to increase the Ct by 4.47 in *P. belbahrii* samples in the absence of PMA, indicating that there was less DNA present after heating, or that the DNA was less amenable to PCR amplification. Addition of PMA to both live (untreated) and dead

(heat-treated) samples also resulted in an increase in Ct (3.03 and 7.22, respectively), compared to samples not receiving PMA, highlighting the need to account for the reductive effect of the compound on DNA in the sporangial samples. The increase in Ct caused by heat-treatment and addition of PMA highlighted the need to prepare sub-samples of sporangia for each condition, to ensure results could be interpreted correctly.

Quantification of a serial dilution of DNA extracted from the live and dead *P. belbahrii* spores using identical qPCR conditions indicated pathogen DNA could each be quantified accurately from 1 x 10^6/ml to a level equivalent of 1 x 10^2 spores/ml using the PMA approach, based on the highest Ct value observed in dead cells.

Heat-treatment of *P. effusa* sporangia (1 x10^6/ml) resulted in a significant increase of Ct 6.19 in the dead compared to live, untreated samples (Table 13) using the PMA-qPCR approach. The increase in Ct indicated more than a 15 x reduction in the quantity of *P. effusa* DNA in the heat-treated samples, compared to the live, untreated samples. This suggested that heat-shock at 95°C for 5 mins had a potentially stronger negative effect on *P. effusa* sporangia compared to *P. belbahrii.* An average difference in Ct of 6.17 was observed between samples comprising the serial dilution prepared from DNA extracted from the live and dead 1 x 10^6/ml spore suspension.

Table 13. Cycle threshold (Ct) values for DNA extracted from live and dead *P. effusa* spores treated (+) or untreated (-) with PMA dye (1x 10^6/ml), and serial dilution of samples mimicking spore suspensions 1x 10^5 to 1 x 10^2/ml.

***P. effusa* spores (ml)**	**Sample**	**Ct Mean**	**Ct +/- PMA**	**Ct Live vs Dead**
1 x 10^6	A1-SYBR	7.514571		
	A1+SYBR	8.868258	1.35	
	D1-SYBR	11.42051		
	D1+SYBR	18.96772	7.54	6.19
1 x10^5	A2-SYBR	9.851547		
	A2+SYBR	11.29662	1.45	
	D2-SYBR	14.26445		
	D2+SYBR	21.88094	7.62	6.17
1 x10^4	A3-SYBR	14.6997		
	A3+SYBR	16.00337	1.30	
	D3-SYBR	18.18425		
	D3+SYBR	26.07249	7.89	6.58
1 x10^3	A4-SYBR	17.56918		
	A4+SYBR	18.94994	1.38	
	D4-SYBR	22.23697		
	D4+SYBR	29.73276	7.49	6.11
1 x10^2	A5-SYBR	20.689		
	A5+SYBR	21.930	1.24	
	D5-SYBR	25.437		
	D5+SYBR	32.493	7.06	5.82

Heat treatment was observed to increase the Ct by an average of 3.89 in *P. effusa* samples in the absence of PMA, indicating that there was more than 10x less DNA after heating. Addition of PMA to both live (untreated) and dead (heat-treated) samples also resulted in an increase in Ct, compared to samples not receiving PMA. The increase in Ct on addition of PMA to live *P. effusa* samples was observed to average 1.34, and 7.51 for dead cells; addition of PMA resulted in less dramatic increases of Ct in live *P. effusa* samples compared to live *P. belbahrii*.

Quantification of a serial dilution of DNA extracted from the live and dead *P. effusa* spores using identical qPCR conditions also indicated the pathogen could be quantified accurately from 1 x 10^6/ml to a level equivalent of 1 x 10^2 spores/ml using the PMA approach, in a similar manner to *P. belbahrii*.

Construction of a draft genome assembly for *P. matthiolae* and basic phylogenetic analysis

A draft genome assembly was constructed for *P. matthiolae*; this measured 84 Mb in length, with a coverage of 61 x. As an estimate of assembly quality, the recovery of single copy orthologs expected to be present in stramenopiles was assessed using BUSCO (Benchmarking Single Copy Orthologs) software (Simao *et al.* 2015); 98% of single-copy genes could be identified, indicating a high-quality assembly. The *P. matthiolae* genome was similar in size to the closely related species *H. arabidopsidis* (88 Mb; Baxter *et al.,* 2010). Approximately 56% of the *P. matthiolae* genome was observed to be repetitive sequences compared to 42 % in *H. arabidopsidis*. Downy mildew of *M. incana* has previously been reported to be caused by *Peronospora parasitica* (syn. *H. parasitica;* Koike *et al.,* 2000), but there are also reports of a separate species, termed *Peronospora matthiolae* (Jafar*,* 1963) being responsible for the disease. We compared genetic distances calculated on differences in house-keeping gene sequences from various downy mildew species to investigate how closely related they were (Results for Beta-tubulin gene are displayed in Figure 17). Sequences from *Hyaloperonospora brassicae, H. parasitica* and *H. arabidopsidis* were included in the analysis. The putative *P. matthiolae* appeared to be most closely related to *H. arabidopsidis* based on the sequences that were compared, also indicating it was a different species to *P. parasitica*.

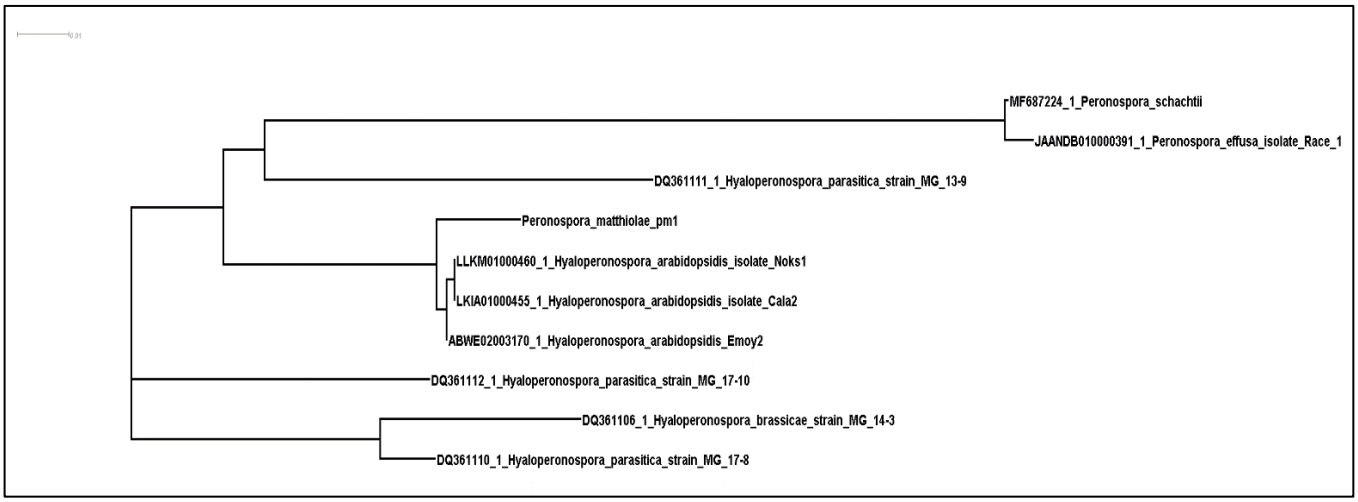

Figure 17. Neighbour joining tree comparing genetic distances based on Beta-tublin coding sequences from *P. matthiolae* and a number of other downy mildew species.

Quantitative PCR testing for the presence of *P. matthiolae* in commercial *M. incana* seed-lots

Six independent lots of *Matthiola incana* seed were tested using a SYBR green quantitative PCR designed to specifically detect *Peronospora matthiolae.* All six *M. incana* seed lots (CP12 -17; Table 14) tested positive for *P. matthiolae.* Average Cts >35 were observed in at least one replicate sample for seed lots CP12, 13, 16 and 17, despite these being below the threshold of detection (Ct 35). However, signal was not generated for all the replicate samples, indicating potentially no *P. matthiolae* was present. Overall, this finding indicated low levels of pathogen DNA in the four respective lots and many were below the threshold of detection, despite still possibly posing a risk. Average Cts of 30.8 and 30.2 were observed for lots CP 14 and 15, respectively, indicating a greater amount of *P. matthiolae* DNA was present on/in the seed compared to the other four lots tested. However, a Ct of ~30 indicates there is <100 fg of *P. matthiolae* DNA, equivalent to < 1 genome (1 Gb DNA= ~ 1 pg; *P. matthiolae* has an estimated genome of 83 Mb (Webb, in preparation)). Thus, even seed with the highest Ct presented a potentially low level of infection.

Table 14. Cycle threshold (Ct) values for detecting *P. matthiolae* DNA extracted from six *M. incana* seed-lots.

Flower Colour	Coded lot	Ave. Ct (40)	Positive tests (9)
Blue	CP 12	36.67399	3
Yellow	CP 13	35.6	1
White	CP 14	30.78334	9
Lavender	CP 15	30.24626	9
Yellow	CP 16	36.11018	5
Pink	CP 17	36.22213	6

Discussion

Fungicide resistance tests were restricted by the availability of fresh isolates from current epidemics. Nevertheless, tests were successfully carried out in 2019 and 2021 on *B. lactucae* isolates from lettuce, and on *P. matthiolae* from column stocks and in 2021 on *P. effusa* isolates from spinach.

These tests generally gave excellent levels of control with Mandipropamid, Dimethomorph and Azoxystrobin against *B. lactucae* and *P. effusa* isolates. With *B. lactucae,* Mandipropamid (Revus) gave consistently high levels of disease control at field rate (99-100%) across all isolates tested, whilst, Dimethomorph (95-100%) and Azoxystrobin (90-100%) showed good control of lettuce downy mildew at the highest rates tested (equivalent to field application rates). In the calculation of EC_{50} values for longer-term reference, some large differences in the min and max values were noted for some isolates, particularly for Dimethomorph and Azoxystrobin. These individual values need to be treated with caution – their true value/meaning will only emerge with longer-term screening and calculations of EC_{50} values. Similarly, with *P. effusa*, all three fungicides gave high levels of control when applied at 'field rates'; Mandipropamid (100%), Dimethomorph (100%) and Azoxystrobin (99-100%). In this case calculation of EC_{50} showed small differences between isolates' reactions to all 3 products tested but these differences were not shown to be statistically significant, showing control in the small population of *P effusa* tested so far to be consistent.

Tests gave variable and less clear-cut control with both Mandipropamid and Dimethomorph against *P. matthiolae* isolates than against those of *B. lactucae* and *P. effusa*. With Manidipropamid generally good results were achieved although better control was seen in 2019 (80-86%) than in 2021 (52-82%). With Dimethomorph there appeared to be a progression towards more widespread resistance with 80-90% control in 2019 declining to 10-50% in 2021. Azoxystrobin was not tested, whilst Metalaxyl was excluded from 2021 tests after *P. matthiolae* isolates showed widespread resistance to it in 2019. There was variable and generally moderate disease control with Fosetyl-Aluminium (28-68%) in both years, and moderate and variable control was also seen with Amectotradin/Dimethomorph (34-66%) and Mancozeb (15-65%) in 2021. It seems unlikely that in the short to medium term Metalaxyl used alone will achieve downy mildew control in column stocks, whilst both Fosetyl-Aluminium and Amectotradin/Dimethomorph will need to be used with caution.

Viability qPCR has been demonstrated to be effective in detecting differences in the amount of DNA in live and dead cells from *P. belbahrii* and *P. effusa*. A difference of Ct 4.04 was observed between live and dead samples containing *P. belbahrii* spores and a Ct 6.19 for *P. effusa* spores (1×10^6/ml).

We observed that it was possible to detect viable DNA at a level equivalent ~10 spores per ml for both *P. belbahrii* and *P. effusa*. The threshold for accurately interpreting qPCR data is Ct 35, and although this is close to the average Ct value generated for 'Dead + PMA cells' observed when testing *P. belbahrii* DNA equivalent 1 x 10^2 spores, it should be possible to detect to the level of a single spore if specificity can be validated through the melt curve profile. This will need to be confirmed through testing on live sporangia, but the hypothesis is supported by the fact qPCR assays can accurately detect sub-genomic quantities of DNA extracted from contaminated seed samples and thus should be effective.

The average difference in Ct between live and dead cells in the complementary dilution series was Ct 4.19 for *P. belbahrii* and Ct 6.17 for *P. effusa.* This difference was most likely due to differences in the effects from the heat-treatment and addition of the PMA to the spore samples. Heat treatment increased the Ct by 4.47 in *P. belbahrii* samples in the absence of PMA, indicating that there was DNA present that was amenable to PCR amplification. This could have been caused by binding of cell-wall components to the DNA, degradation of the DNA after sporangial-lysis, influence of the cell contents on taq polymerase, or a combination of these factors. The difference in Ct caused by the heat-treatment alone highlights the necessity to include dead-cell controls. Heat-treatment appeared to have less effect on DNA levels in *P. effusa,* with an increase of Ct 3.89 between live and dead cells in the absence of PMA.

Addition of PMA to live (untreated) samples of *P. belbahrii* and *P. effusa* spores resulted in an increase in Ct 3 and Ct 1.44, respectively, compared to samples not receiving PMA. This indicated that addition of PMA had a greater effect on reducing DNA content in *P. belbahrii* compared to *P. effusa* and could have contributed to the reduced sensitivity of the assay. A difference in the effect of PMA on the dead (heat-treated) samples was less obvious with an average Ct 7.22 observed for *P. belbahrii* and Ct 7.51 for *P. effusa.* These observations highlighted the importance of including controls for the various experimental conditions to account for the effects of the PMA and heat-treatment when estimating the differences in Ct between live and dead cells.

Overall, the approach was effective at determining differences in the amount of viable DNA in quantities that would be expected in contaminated lots, however the amount of control assays required for implementing the testing and requirement for fresh downy mildew spores, necessitates a lot of preparation and costs more when compared to reverse transcription qPCR methods that target RNA/active gene expression.

A draft genome assembly was constructed for *P. Matthiolae* utilising Oxford Nanopore Technologies MinION platform and subsequently annotated to provide information on the genetic content. Genetic sequence data was utilised to conduct basic phylogenetic analysis

to investigate how closely related *P. matthiolae* was to a number of major downy mildew species, in order to confirm the identity of the pathogen isolated from symptomatic column-stock plants. Previous reports indicated that downy mildew of stocks can be caused by *P. matthiolae* and *P. parasitica (*syn. *H. parasitica).* It was clear from the analysis that the mildew isolated from column stock was more closely related to *H. arabidopsidis* than *H. parasitica/H. brassicae.* Further investigation will be required to assess if *P. matthiolae* is able to infect Brassica hosts and likewise if *H. parasitica/H. brassicae* can successfully infect *M. incana.*

Quantitative PCR was conducted on a series of six commercial *Matthiola incana* seed lots that were suspected of having downy mildew contamination, using a novel assay designed for the project. This is the first time a diagnostic assay has been described for the detection of *P. matthiolae* in seed. *P. matthiolae* was detected in all the six seed lots tested, but only two were found to contain consistent levels of DNA throughout the three replicated extracts. The remaining four contained only negligible quantities of pathogen DNA and not all replicate samples tested positive, indicating *P. matthiolae* was only present at relatively low levels. Replicate samples used for the DNA extractions comprise of 50 seeds, so increasing seed number to 100 could potentially help to increase sensitivity, however a doubling of DNA content would only reduce Ct by ~1.5 and the quality of the extract would likely to be compromised. Given the small quantities of *P. matthiolae* DNA detected in the six seed lots tested and verified reports of disease arising from them, even low levels of the pathogen may pose a threat to growers. Further effort will be required to assess if PMA or RT qPCR can be utilised to detect the presence of viable *P. matthiolae* in seed, and the risk this could present..

Conclusions

- Review has shown that 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 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, although 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.

- Molecular testing was demonstrated capable of detecting and quantifying small amounts of basil, spinach and column stock downy mildew (DNA/RNA) both externally (in basil and spinach seed-lots) and inside contaminated seeds of basil, spinach and stocks.
- LAMP primers have been designed for *Bremia* and have been successfully tested for specificity and efficacy in LAMP qPCR.
- Seed-lots of basil, spinach and column stocks containing very low levels of downy mildew (DNA/RNA) still potentially pose a high disease risk to growers.
- Across the seed-lots of basil and spinach tested, a greater quantity of downy mildew DNA was detected inside the seed coat compared to seed washings.
- Steam-treated basil seed lots contained approximately 50% less downy mildew DNA than untreated samples from identical lots, indicating that steam-treatment reduces the pathogen load.
- The downy mildew isolated from the column stock samples tested in this study was confirmed as *Pernospora matthiolae* by both morphological characters, and nucleotide sequence analysis, and basic phylogenetic study showed this to be more closely related to *Hyaloperonospora arabidopsidis* than *H. parasitica/H. brassicae.*
- Quantitative PCR was conducted on commercial *Matthiola incana* seed lots suspected of containing downy mildew contamination, using a novel assay designed for the project. This is the first time a diagnostic assay has been described for the detection of *P. matthiolae* in seed.
- Thirty-nine lettuce *Bremia lactucae* isolates were collected from 2019-2021 and assessed for race structure according to IBEB guidelines and protocols kindly supplied by Naktuinbouw, who also supplied seed of the 16 current accessions in the official lettuce differential set (Set C). Twenty-eight putative races were identified and of these, one (2020_BL4G) matched IBEB committee race description Bl:24EU, whilst four others (2019_BL2A, 2019_BL2B, 2021_BL11A & 2021_BL11C) matched IBEB race Bl:35EU. Comparisons of this data with publicly available data will continue after the end of this project, whilst race testing results for all of the isolates tested from 2019 to 2021 were included in the IBEB EU Groslist 2021.

- Viability qPCR using Propidium Monoazide PMA has been demonstrated to be effective in detecting differences in the amount of DNA in live and dead cells from *P. belbahrii* and *P. effusa*. This procedure can be used effectively to determine the presence of viable/no-viable cells of *P. belbahrii* or *P effusa* in infected seed-lots, although research in this project as shown that control assays are needed to account for the internal effects of heat and of PMA addition which require the generation and use of live spores for every test, necessitating a lot more preparation than originally anticipated, and therefore higher costs when compared to reverse transcription qPCR methods that target RNA/active gene expression.
- Fungicide resistance tests at JHI were restricted by the availability of fresh isolates from current epidemics. Nevertheless, tests were successfully carried out in 2019 and 2021 on *B. lactucae* isolates from lettuce, and on *P. matthiolae* from column stocks and in 2021 on *P. effusa* isolates from spinach (AHDB CP184 reports 2019 and here).
    - Fungicide resistance tests on *B. lactucae* isolates obtained from the industry in 2019 and 2021 showed that:
        - Mandipropamid (Revus) gave consistently high levels of disease control at field rate (99-100%) across all isolates tested.
        - Dimethomorph (95-100%) and Azoxystrobin (90-100%) also showed good control of lettuce downy mildew at field application rates.
        - These tests were carried out for the calculation of EC50 values for longer-term reference and use alongside pathogen race identifications, some large differences in the min and max values were noted for some isolates, particularly for Dimethomorph and Azoxystrobin. These individual values need to be treated with caution – their true value/meaning will only emerge with ongoing longer-term screening and EC50 calculations.
    - Fungicide resistance testing carried out on *P. matthiolae* isolates by JHI in 2019 and 2021 had the following outcomes:
        - Widespread resistance to Metalaxyl was found across a range of isolates in 2019 with no further testing therefore carried out
        - Fosetyl-Aluminium gave moderate disease control (28-68%) in 2019 and 2021
        - Mandiprompamid gave better control in 2019 (80-86%) than 2021 (52-82%)

        - The tests suggest resistance to Dimepthomorph may be developing given the 80-90% control found in 2019 dropped to 10-15% in 2021
        - Amectotradin/Dimethomorph and Mancozeb gave moderate and variable control of 34-66% and 15-65% respectively in 2021
    - Fungicide resistance testing at JHI on P. effusa isolates in 2021 showed that:
        - When applied at field rate, Mandipropamid and Dimethomorph gave 100% control
        - Azoxystrobin applied at field rate gave excellent control between 99-100%.
        - Calculation of EC50 showed small differences between isolates' reactions to all 3 products tested but these differences were not shown to be statistically significant, showing control in the small population of P effusa tested so far to be consistent.

- Review of general downy mildew biology and of Decision Support Tools (DST) to assist for their integrated management 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. Wood, T (2020) Oral presentation: *Seed-borne inoculum for basil downy mildew: detection, incidence and transmission.* Basil Downy Mildew Workshop, Stockbridge Technology Centre, Cawood 5th March 2020.

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

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

- Pettitt, T. (2020) Oral presentation: *Oomycete diseases control – guiding principles and practical application.* AHDB Webinar 'Prevent, detect and control oomycete crop diseases', 23rd July 2020. https://ahdb.org.uk/events/prevent-detect-and-control-oomycete-crop-diseases

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

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

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