INVESTIGATION OF BASIL DOWNY MILDEW PATHOGEN SURVIVAL, NEW PATHOTYPE DEVELOPMENT AND SOURCES OF QUANTITATIVE

Kelly S. Allen  
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INVESTIGATION OF BASIL DOWNY MILDEW PATHOGEN SURVIVAL, NEW PATHOTYPE DEVELOPMENT AND SOURCES OF QUANTITATIVE DISEASE RESISTANCE

A Dissertation Presented

by

KELLY SUSAN ALLEN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Plant Biology Graduate Program
INVESTIGATION OF BASIL DOWNY MILDEW PATHOGEN SURVIVAL, NEW PATHOTYPE DEVELOPMENT AND SOURCES OF QUANTITATIVE DISEASE RESISTANCE

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DEDICATION

This work is dedicated to the many scientists and plant practitioners who have pioneered the field of plant pathology and endeavored to understand her countless mysteries, and to those who will continue to do so, it is my privilege to work with you.
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Basil downy mildew (BDM) caused by the oomycete pathogen *Peronospora belbahrii*, threatens sweet basil (*Ocimum basilicum*) production worldwide. Chemical and cultural control options for BDM are limited, and resistant cultivars have only recently become available for commercial production. To address this challenging agricultural disease, this research investigates BDM epidemiology, occurrences of new pathotypes, and molecular plant-pathogen interactions leading to host resistance or susceptibility.

A reproducible low-resource inoculation protocol was developed to harvest *P. belbahrii* inoculum and propagate BDM for further research. The survival of *P. belbahrii* sporangia was examined using an *in vitro* assay to assess germination potential and a plant infection bioassay to determine infection potential following exposure to two
temperatures and two humidity levels. Detached *P. belbahrii* sporangia remained viable up to 12 days when kept isolated at 20°C in 96.5% relative humidity, significantly longer than had previously been reported.

The inoculation procedure was utilized to collect unique *P. belbahrii* isolates and perform the first controlled-environment differential cultivar trials to identify new pathotypes of BDM. Two new and distinct pathotypes of BDM were identified, one overcoming cultivars with quantitative resistance, and the second overcoming qualitative resistant ‘Prospera’ lines. Interestingly, quantitatively resistant ‘Mrihani’ and ‘Passion’ were more resistant to the second pathotype, indicating that this pair may hold a unique and important source of quantitative resistance.

The mechanisms of quantitative disease resistance were explored to identify specific genes as molecular markers for breeding using comparative transcriptomics. Candidate gene MRI-R1 was significantly upregulated in BDM-resistant ‘Mrihani’ following pathogen inoculation and is a member of the canonical resistance gene NB-LRR family. Sequencing of MRI-R1 clones identified four alleles, and two of the identified alleles were isolated from two resistant offspring from the genetic cross using ‘Mrihani’ and BDM-susceptible ‘Newton’ as breeding parents. MRI-R1 likely contributes to quantitative resistance against BDM, and further studies will determine the utility of this gene as a breeding marker.

Taken together, these studies have added to the current understanding of BDM epidemiology, the identification of new pathotypes overcoming host resistance, and the identification of candidate genes and mechanisms of quantitative resistance.
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CHAPTER 1

BACKGROUND

1.1 Downy mildews

Abiotic and biotic stress limits yield of agricultural crops, and biotic pathogens account for substantial crop losses each year (Palmer et al., 2017). Foliar vegetable and herb crops including lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), and basil (*Ocimum basilicum*) have been particularly threatened by downy mildew pathogens, biotrophic fungal-like organisms which infect and colonize living foliar tissue (Crandall et al., 2018). These pathogens require periods of high relative humidity in order to infect plant hosts and proliferate (Djalali Farahani-Kofoet et al., 2012; Cohen et al., 2017). Downy mildew epidemics can be difficult to disrupt in part because symptoms and signs of disease, including interveinal chlorosis and visible sporulation from stomata, occur only after infection and pathogen proliferation in the plant tissue has advanced, typically no earlier than 4-5 days post-inoculation. Therefore, research in control methods, resistance breeding, and pathogen population structure has been of increasing interest (Crandall et al., 2018). Recent emergence of downy mildew strains capable of overcoming genetic resistance in hosts, and fungicide-resistant strains have threatened many susceptible crops, particularly lettuce and spinach, and increasingly basil (Cohen et al., 2013; Crandall et al., 2018; Ben-Naim & Weitman, 2021). Effective control options for downy mildew management are limited, particularly in organic production systems (Wyenandt et al., 2015; Crandall et al., 2018). Organic chemical control options have been variably effective, and both organic and conventional chemical control options risk
pathogen strain evolution (Cohen et al., 2013; Pyne et al., 2014; Wyenandt et al., 2015; Cohen & Rubin, 2015).

Downy mildew pathogens require living host tissue to complete their asexual infective life cycle, which results in emergence of sporangiophores bearing sporangia. These pathogens are obligate parasites, as are powdery mildew and rust fungi, and cannot be cultured and maintained axenically (Koch & Slusarenko, 1990). Significant advances have been made in understanding the host-pathogen interactions of downy mildews and other obligate parasite pathogens using the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Coates & Beynon, 2010).

### 1.2 Basil downy mildew

Sweet basil (*Ocimum basilicum*) is one of the most important culinary and essential oil herbs grown in the US and globally (Wyenandt et al., 2015). Basil downy mildew (BDM), caused by the pathogen *Peronospora belbahrii*, is the most significant disease threatening sweet basil production worldwide (Wyenandt et al., 2015; Cohen et al., 2017). Due to BDM, basil growers have consistently reported up to 100% losses of susceptible varieties, estimated to be worth tens of millions of dollars (Roberts et al., 2009; Wyenandt et al., 2015).

First introduced to the United States and the Northeast in 2007, BDM is re-introduced each growing season via inoculum traveling north on southerly winds, or through infected transplants and rarely through seed (Wyenandt et al., 2015; Cohen et al., 2017). As of 2021, BDM has been reported in 44 U.S. states (Figure 1.1).
BDM is difficult to manage due to limited control options, exacerbating the need for resistant basil cultivars (Wyenandt et al., 2015; Crandall et al., 2018). Multiple fungicide efficacy trials have suggested that basil downy mildew is best controlled using a preventative spray program with ample coverage of the top and bottom of foliage, but there are currently no organic fungicide options that provide effective control (Mcgrath et al., 2014; Wyenandt et al., 2015; Mcgrath, 2016). Conventional fungicides options are limited and can risk increased pathogen resistance, and the demand for organically grown basil is a priority for consumers.

*Peronospora belbahrii* is an oomycete that infects basil leaf mesophyll tissue by directly germinating and penetrating the leaf tissue or entering through stomatal openings.
of the leaves (Wyenandt et al., 2015; Cohen et al., 2017). The pathogen hyphae spread intercellularly and produce haustoria, nutrient absorption structures, which are invaginated into the plant cells (Wyenandt et al., 2015; Cohen et al., 2017; Zhang et al., 2019). After 4-5 days, symptoms and signs of infection become barely visible, including interveinal chlorosis and gray, mildew-like sporulation on the underside of leaves (sometimes emerging on the adaxial surface as well) (Figure 1.2) (Belbahri et al., 2005; Garibaldi et al., 2007; Koroch et al., 2013; Cohen et al., 2017).

Figure 1.2 Basil infected with downy mildew. A. Whole potted plants with advanced symptoms of interveinal chlorosis and signs of pathogen sporulation on abaxial and adaxial leaf surfaces. B. Detached basil leaf with profuse sporulation on the abaxial surface.

The spore-bearing structures, asexual conidiophores (hereo referred to as sporangiophores), produce asexual conidia (hereo referred to as sporangia) abundantly (Figure 1.3). Infective sporangia are wind dispersed and cause polycyclic infections in both field and greenhouse production settings (Cohen et al., 2017).
Current understanding of the basil downy mildew infection cycle remains incomplete (Wyenandt et al., 2015; Elad et al., 2016; Cohen et al., 2017). The pathogen progresses through asexual reproduction similarly to spinach downy mildew (Peronospora effusa), but the timing of each step appears to be dependent on environmental conditions. Disagreement regarding the timing of sporangia germination, lead to variations in reported timing of the infection process in published studies. An assessment of P. belbahrii sporangia germination in vitro revealed that germination ensued before 4 hours at temperatures under 15°C and occurred between 8-48 hours at temperatures above 20°C (Farahani-Kofoet, 2014). An in vivo study demonstrated that sporangia germinated by 2 hours post-inoculation (Cohen et al., 2017), while a different in vivo characterization using scanning electron microscopy (SEM) of the infection process showed sporangia germination from 2-6 days after inoculation (Zhang, N. et al., 2019). Furthermore, SEM characterization of the infection revealed that sporangia germ
tube development does not appear to be directed toward plant stomata, nor did it change direction as germ tubes grew over stomata, and penetration occurred directly into the plant epidermis (Zhang, N. et al., 2019). Previous reports hypothesized or demonstrated hyphal growth directly into stomata as well as direct penetration into leaf tissue (Cohen et al., 2017; Wyenandt et al., 2015). All studies agree that *P. belbahrii* germinating hyphae were able to penetrate directly through the abaxial and adaxial leaf surfaces, as does *Hyaloperonospora arabidopsidis* (Cohen et al., 2017; Coates & Beynon, 2010; Zhang, N. et al., 2019).

The discrepancies in the literature regarding *P. belbahrii* epidemiology suggest that, apart from the practical difficulties of characterizing the basil downy mildew pathosystem, the infection process is influenced by environmental factors, which vary in naturally occurring infections, as well as in research methodologies.

A comparative transcriptomic study revealed expression of putative cytoplasmic effectors (presumably released into host cytoplasm through the host-pathogen interface of the extrahaustorial matrix) beginning as early as 12 hours post-inoculation and increasing along the time course of infection (data not shown). These data suggest that haustoria development occurs within 48 hours post-infection.

Taxonomic investigation and observation of *P. belbahrii*-like symptoms and pathogen structures on new hosts in the family Lamiales, which includes basil, have led to the conclusion that *P. belbahrii* is a species complex, with several subspecies identified on basil relatives (Henricot *et al.*, 2010; Rivera *et al.*, 2016; Thines *et al.*, 2020; Hoffmeister *et al.*, 2020). One study showed that isolates of *P. belbahrii* infecting basil
collected in Israel were not virulent on Coleus, but were able to infect and sporulate on rosemary (Rosmarinus officinalis), Nepeta (Nepeta curviflora), Clinopodium (Micromeria fruticosa), and two species of sage (Salvia pinnata and S. fruticosa) (Cohen et al., 2017).

1.3 Mechanisms of disease resistance and deployment in plant breeding

Plant resistance to pathogen invasion involves pathogen detection using resistance genes (R-genes) (Glazebrook, 2005; Jones & Dangl, 2006; Palmer et al., 2017). A major class of known plant R-genes encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins (Nelson et al., 2018). Single and/or major R-gene mediated resistance, also known as qualitative or vertical resistance, recognizes pathogen effectors secreted into the plant cell, but can be quickly overcome by the emergence of new virulence races (henceforth referred to as pathotypes) (Durrant & Dong, 2004; Nelson et al., 2018; Spring et al., 2018).

Microbes that have successfully evolved effector proteins are able to suppress the basal plant immune response, known as MAMP-triggered immunity, and elicit effector-triggered susceptibility from plants (Jones & Dangl, 2006). Plant resistance proteins recognize pathogen effectors, and an effector-triggered immune response is rapidly deployed. The zig-zag model of plant-pathogen interactions explains this oscillating interaction of plant defenses and pathogen virulence factors, culminating in the initiation of infection and disease by successful pathogens (Jones & Dangl, 2006). These interactions occur at the cellular interface and elicit systemic responses (Figure 1.4).

Plant hormone signaling by salicylic acid, jasmonic acid, and ethylene is activated during plant resistance responses, and play crucial roles in the success or failure of plant
defense. Salicylic acid synthesis is initiated during both MAMP-triggered immunity and effector-triggered immunity (Asai et al., 2014; Palmer et al., 2017). Salicylic acid is accumulated locally at the infection site of biotrophic and hemi-biotrophic pathogens to induce a hypersensitive response during effector-triggered immunity (Palmer et al., 2017). Virulent biotrophic and hemi-biotrophic pathogens have been found to attenuate the salicylic acid signaling pathway (Glazebrook, 2005; Asai et al., 2014).

Salicylic acid synthesis during pathogen infection also initiates systemic acquired resistance through the production of pathogenesis-related proteins, phytoalexins, and cell wall modifications during effector-triggered immunity (Palmer et al., 2017). Systemic acquired resistance is a broad-spectrum defense response originating from a point inoculation and resulting in systemic signaling and elevation of defenses in distal tissues (Durrant & Dong, 2004). Systemic acquired resistance is regulated by salicylic acid signaling (primarily produced through the isochorismate pathway), which induces activity of the positive regulator NPR1 and leads to increased production of pathogenesis-related proteins (Durrant & Dong, 2004). During systemic acquired resistance, hydrogen peroxide accumulates in distal tissues within 2 hours following hydrogen peroxide microbursts at the point of infection (Durrant & Dong, 2004). Lettuce plants resistant to the lettuce downy mildew pathogen Bremia lactucae also show increased hydrogen peroxide accumulation early after inoculation, leading to a systemic acquired resistance-mediated hypersensitive response (Sedlářová et al., 2011).

Two distinct mechanisms of NB-LRR-mediated plant immunity have been recently elucidated. Arabidopsis NB-LRR protein ZAR1 indirectly recognizes several
pathogen effectors through helper-protein binding, which activates ZAR1 resistosome formation through protein complex pentamerization, membrane association and formation of a calcium ion channel (Wang Jizong et al., 2019; Adachi et al., 2019; Bi et al., 2021). Arabidopsis downy mildew resistance protein RPP1, an NB-LRR with an N-terminal TIR domain, directly recognizes downy mildew *Hyaloperonospora arabidopsidis* effector ATR1 and forms an active tetramerized resistosome that functions as a NAD-hydrolyzing holoenzyme, leading to further downstream effector triggered immunity (ETI) activation by the activation of other protein complexes again leading to calcium ion influx signaling (Krasileva et al., 2010; Ma et al., 2020).
Figure 1.4 A generalized model of the molecular and structural mechanisms involved in pattern triggered immunity, PTI, and effector triggered immunity, ETI. Taken from (Margts et al., 2021). Pathogen effectors (fuchsia) are secreted and localized to the plant apoplast (circles) or cytoplasm (random shapes), where they are directly or indirectly detected by pattern recognition receptors associated with PTI (right) or NB-LRRs associated with ETI (left). Two mechanisms of NB-LRR directed ETI are depicted at the left, with coiled-coil NB-LRRs (CNLs) representing pentamerized resistosome activation and calcium ion channel formation to inducing a hypersensitive response (as in Arabidopsis ZAR1), and TNLs depicting a tetramerized resistosome with NAD-hydrolyzing holoenzyme activity leading to downstream activation of ETI (as in Arabidopsis RPP1).

Plant resistosome-mediated immunity may be a conserved mechanism controlling qualitative resistance when gene-for-gene interactions are defined. Quantitative, multigenic resistance is less understood, but of equal relevance to the practice of breeding for disease resistance. Plant breeders are interested in breeding for quantitative or “durable” resistance controlled by stacking multiple genes with different functions (Mundt, 2018). Quantitative resistance can result in reduced infection by pathogens,
independent of differential virulence pathotypes, and is therefore a more robust trait for breeding disease-resistant crops (Mundt, 2018; Martins et al., 2019). Due to the multigenic nature of quantitative disease resistance, there have been no clear mechanisms defined, but progress is being made toward identifying quantitative disease resistance loci and specific genes for marker-assisted breeding.

Sweet basil is an outcrossing allotetraploid with very limited genomic resources, and breeders are challenged to create new cultivars and/or hybrids that result in fertile progeny with disease resistance while retaining desired horticultural traits (Pyne et al., 2017). Sweet basil ‘Mrihani’ was identified as a BDM-resistant cultivar in the basil breeding program at Rutgers University through a screen of 39 basil accessions representing a broad geographic distribution (Pyne et al., 2014). A quantitative trait locus (QTL) analysis was used to identify one major and two minor loci conferring downy mildew resistance (Pyne et al., 2014, 2015, 2017). ‘Mrihani’ is a unique aromatic cultivar, often grown as an ornamental, but it does not meet the needs of growers looking for BDM-resistant sweet basils. The Rutgers breeding program produced four BDM-resistant sweet basil cultivars from ‘Mrihani’ crossed with BDM-susceptible ‘Newton’ (known as SB22 in the Rutgers breeding program) (See APPENDIX A), and additional resistant cultivars have been produced in other breeding necessitating the need for continued development of increasingly and diversely resistant cultivars. Quantitative resistance loci are complex, sometimes containing multiple linked genes with related functions, and those that have been fine-mapped and cloned include NB-LRRs, pattern recognition receptors, receptor-like kinases, and other classes often involved in defense response pathways (Nelson et al., 2018). The linkage mapping performed in the
‘Mrihani’ X SB22 F₂ mapping population suggested the action of one or more dominant genes controlling resistance (Pyne et al., 2017).

In addition to identifying plant R-genes and loci, identification of pathogen effectors involved in both virulence and avirulence responses is an important field of study. Plant pathogens use effectors to manipulate plant defenses (Hein et al., 2009; Sperschneider et al., 2015) and RXLR (Arg-Xaa-Leu-Arg) motif-containing proteins are the classical examples of effectors involved in pathogenesis among oomycete plant pathogens, including downy mildews (Win et al., 2007; Oh et al., 2009; Wawra et al., 2012; Vleeshouwers & Oliver, 2014). Canonical RXLR effectors suppress or activate common plant defense responses, including hypersensitive cell death (Hein et al., 2009). Because of these interactions with the host, pathogen effectors can be effectively utilized as “baits” for identification of new sources of genetic resistance (Goritschnig et al., 2012; Vleeshouwers & Oliver, 2014). Furthermore, pathogen effector sequence disordering has been identified as a mechanism by which pathogens can evade recognition by cognate plant R-genes, demonstrating the importance of introducing durable multi-genic resistant cultivars into production (Yang et al., 2020). Using the conserved RXLR motif (Wawra et al., 2012; Delulio, 2018), we have identified 11 candidate RXLR effectors involved in BDM (data not shown). The following studies do not address these important pathogen genes but provide the necessary groundwork to understand how differential virulence in response to varying sources of resistance are linked to effector diversity.
1.4 References


Ben-Naim Y, Weitman M. 2021. Joint action of Pb1 and Pb2 provide dominant complementary resistance against new races of Peronospora belbahrii (Basil Downy Mildew). *Phytopathology®*: PHYTO-02-21-0065-R.


CHAPTER 2

EVALUATION OF *PERONOSPORA BELBAHRII* SPORANGIA SURVIVAL AND PATHOTYPE VIRULENCE PATTERNS ON BASIL CULTIVARS

2.1 Introduction

Basil downy mildew is one of several downy mildew diseases impacting culinary herb production globally, which can rapidly result in total crop loss of susceptible varieties with estimated economic impacts in the tens of millions of dollars (Roberts *et al.*, 2009; Wyenandt *et al.*, 2015; Hoffmeister *et al.*, 2020). Basil downy mildew is caused by *Peronospora belbahrii*, an oomycete pathogen that infects basil leaf mesophyll tissue by penetrating leaf tissue or entering through stomatal openings on the lower surface of the leaves (Cohen *et al.*, 2017). Symptoms and signs of infection include interveinal chlorosis and gray, mildew-like sporulation on the underside of leaves (sometimes emerging on the adaxial surface as well) (Belbahri *et al.*, 2005; Garibaldi *et al.*, 2007; Koroch *et al.*, 2013). Sporangioaphores, the spore-bearing structures that emerge from stomata of infected plants, produce abundant sporangia which are wind-dispersed in both field and greenhouse production settings (Cohen *et al.*, 2017).

Understanding the disease cycle for the control of any plant pathogen requires a thorough understanding of the factors involved in the infection process (Berger, 1977). The traditional concept of the plant disease triangle organizes the factors required for disease into three broad categories: a virulent pathogen, a susceptible host, and a favorable environment (Francl, 2001). As our understanding of plant disease has increased, an improved disease tetrahedron model was proposed to further incorporate a fourth broad category accounting for biotic interacting factors, such as endophytic
species, vectors, etc. (Brader et al., 2017). In the case of BDM, the disease cycle starts with *P. belbahrii* sporangia. The establishment of the disease requires the germination of virulent sporangia and then penetration of susceptible plant leaves under favorable environmental conditions to establish the infection.

The effects of environmental factors, such as the relative humidity (RH), temperature, and dew period on the sporulation and the initiation of infection by the pathogen *P. belbahrii* have been well-described over the past several years (Cohen et al., 2013a, 2017; Cohen & Rubin, 2015; Cohen & Ben-Naim, 2016). Infection requires more than 4 hours of leaf wetness duration (i.e. free water present on the leaf surface) for *P. belbahrii* sporangia to germinate and penetrate plants at temperatures between 15-20°C (Rowlandson et al., 2015; Cohen & Ben-Naim, 2016; Cohen et al., 2017). Sporangia germination and plant infection only occurs between a minimal 5°C and maximal 28.5°C, in the dark under a 8-12 hour dew period (Cohen et al., 2017). These temperature ranges decrease with shorter dew periods (Cohen et al., 2017). The maximum infection was reported at temperatures between 11.5 to 15.5°C with 8 hours of dew period (Cohen et al., 2017). Following leaf penetration by infective sporangia, intercellular hyphal colonization occurs at 8.5-30°C during 12 hour photoperiods, with optimal daytime temperatures of 20-25°C (Cohen et al., 2017).

Pathogen sporulation perpetuates the polycyclic disease cycle, and is the most important factor in disease initiation, progression and spread. The sporulation process can occur as early as four days post-infection, depending on the density of the initial infection and the environmental conditions (Wyenandt et al., 2015; Cohen & Ben-Naim, 2016). Even though no free leaf moisture is required for the sporulation to occur, a period of
high RH is required and the temperature range of sporulation is dependent on the duration of the dew period (Cohen et al., 2017). For example, 20 hours of 100% RH was reported to induce sporulation at 18°C in the dark (Cohen et al., 2017). Sporangia production decreased significantly when infected detached leaves were incubated at <94.60% RH compared to >97.60% RH (Cohen & Ben-Naim, 2016). Detached leaves incubated at 100% RH and 97.59% RH produced an average of ~8.75x10^5 and ~8.125x10^5 sporangia per leaf, respectively, while incubation at 94.60% RH reduced sporulation to ~2.5x10^5 sporangia per leaf (Cohen & Ben-Naim, 2016). Sporulation dropped to almost zero at 85% RH and was nullified at 75.50% RH (Cohen & Ben-Naim, 2016).

Sporangiophores were reported to first emerge from the stomata of infected leaves after approximately 3 hours during incubation in a 100% RH dew chamber at 18°C in the dark (Cohen & Ben-Naim, 2016). Sporangiophores developed branches beginning at 4 hours of incubation and continuing through 6 hours. By 8 hours of incubation, sporangia begin to develop and the maximum sporulation occurred at 11-14 hours following incubation (Cohen & Ben-Naim, 2016; Cohen et al., 2017).

The survival of sporangiophore-attached and detached sporangia was found to be strongly influenced by temperature, and survival of detached spores was found to be abolished after a maximum of 96 hours at 25°C (Cohen & Rubin, 2015). A previous study based on plant inoculation bioassays suggested that detached sporangia kept at minimum temperatures of 25°C were unable to infect plants after 96 hours (Cohen et al., 2017). Based on the demonstrated survivability of sporangia and observed patterns of disease spread, it is believed that sporangia cannot overwinter in Northern climates (Wyenandt et al., 2015; Cohen et al., 2017).
These epidemiological studies had a critical impact on identifying effective cultural control strategies to reduce downy mildew infections, including the implementation of nocturnal fanning, solar heating, and nighttime illumination to inhibit sporangia development (Cohen et al., 2013a, 2017; Cohen & Rubin, 2015; Cohen & Ben-Naim, 2016).

Basil downy mildew was first introduced to the United States and the Northeast in 2007, and has been continually active in the south or re-introduced each growing season via aerially-dispersed inoculum or infected transplants (Roberts et al., 2009; Wyenandt et al., 2015). Seed transmission of the pathogen was reported, but unable to be replicated in controlled trials (Djalali Farahani-Kofoet et al., 2012; Cohen et al., 2017; Falach-Block et al., 2019). By 2021, 44 U.S. states reported downy mildew infections, some over multiple years (McGrath, 2021), and there is currently no policy in place for controlling shipment of infected material. While in previous years basil downy mildew only became active in the Northeast later in the growing season (August-September), in June of 2021 and 2022, downy mildew-infected plants were being sold in garden centers in Massachusetts (reported in https://basil.agpestmonitor.org/). These early introductions of active inoculum escalate the timeline and challenges for Northeast growers to control the disease.

In controlled-environment settings, dispersed sporangia may be released onto greenhouse benches, potting materials, and other surfaces. However, a previous study based on plant inoculation bioassays suggested that detached sporangia kept at minimum temperatures of 25°C were unable to infect plants after 96 hours (Cohen et al., 2017). Destruction of an infected crop following a disease outbreak in the greenhouse or the
field is a way to prevent further disease spread (McGrath et al., 2014; Wyenandt et al., 2015; McGrath, 2016). While oospores, produced after sexual reproduction of oomycetes, have been observed in mesophyll tissue, oospore-infested soil did not produce infection in new plantings (Cohen et al., 2013b, 2017) and the role of sexual reproduction in survival of the pathogen remains unclear. Therefore, the emphasis is on the control of sporangia, which may be released onto greenhouse benches, stray pots, or soil leading to further infections. It is thus critical to understand the survivability of sporangia as a direct indicator of pathogen infectivity. One aspect of sporangia survival that remained undetermined was maximum survivability at optimal temperatures and extended periods of RH. Simply put, how long could sporangia be expected to survive after dispersal under optimal environmental conditions?

In addition to targeting environmental factors and interrupting pathogen epidemiological processes, improving plant resistance is another critical component of controlling disease (Brader et al., 2017; Francl). Sweet basil is a challenging species to breed, but there have been multiple introductions of downy mildew resistant (DMR) cultivars since 2018. These resistant cultivars include the Rutgers DMR lines ‘Devotion’, ‘Obsession’, ‘Passion’, and ‘Thunderstruck’, which derive quantitative resistance from ‘Mrihani’ (MRI) (Mcgrath et al., 2014; Pyne et al., 2015, 2017) (APPENDIX A). A different breeding group developed a line of resistant cultivars called ‘Prospera’ with qualitative (or major gene-mediated) resistance by introgression of highly resistant O. americanum accessions with susceptible sweet basil ‘Peri’ (Ben-Naim et al., 2018). The ‘Prospera’ lines carry qualitative resistance controlled by a pair of dominant genes (Pb1A and Pb1A’) (Ben-Naim & Weitman, 2021). Other resistant cultivars released
include ‘Amazel Basil®’ (Proven Winners, USA), which is a sterile hybrid with qualitative resistance derived from the same *O. americanum* accession as the ‘Prospera’ lines, and recent introduction ‘Pesto Besto™’ (Proven Winners, USA), which carries the same source of resistance as ‘Amazel Basil®’ but can be grown from seed (Ben-Naim & Weitman, 2021).

The introduction of resistant cultivars has predictably been accompanied by reports of breakdown of resistance or new, more virulent pathogen strains. The development of new “virulence races” (also referred to as pathotypes) overcoming host resistance has been observed in lettuce downy mildew, spinach downy mildew, and other high impact pathosystems in response to the introduction of resistant cultivars (Irish *et al.*, 2003; Feng *et al.*, 2018; Spring *et al.*, 2018).

The first spinach downy mildew pathogen (*Peronospora effusa*) pathotype overcoming a resistant cultivar was reported in the US and Europe in 1824 (Irish *et al.*, 2003). In 1996, there were five pathotypes identified and by 2018, nineteen individual pathotypes, with differential virulence signatures on spinach cultivars (Irish *et al.*, 2003; Feng *et al.*, 2018) have been denominated by the International Working Group on *Peronospora* in spinach (IWGP). Up until 2021, *P. belbahrii* pathotype differentiation was anticipated but not determined. In 2021, two pathotypes of *P. belbahrii* were described in Israel based on differential virulence of the isolates infecting basil with two different sources of resistance, and can be confidently classified as races by their differential molecular signatures established by simple-sequence repeat, SSR, profiling (Ben-Naim & Weitman, 2021).
Prior to this study, cultivar trials have been performed in field settings using natural inoculum, and disease incidence and severity was assessed weekly to establish the area under the disease progress score (Patel et al., 2021). The limitation of these trials is that they may or may not include a full “differential” set of cultivars to assess whether new pathotypes of *P. belbahrii* have emerged, and natural inoculation introduces a level of variation.

The main objectives of this research are to address the challenges of performing controlled inoculations and collections of *P. belbahrii* to study pathogen epidemiology and pathotype development to improve disease control. Specifically, I developed a reproducible and low-resource inoculation protocol, established a system to evaluate sporangia survival in the absence of host plant material, and identified differential *P. belbahrii* virulence patterns on cultivars to monitor pathotype development.

These studies establish the long-term survival potential of *P. belbahrii* sporangia to inform safe re-entry periods for resuming basil production, and more rigorously defines the differential virulence status of three *P. belbahrii* isolates under controlled environmental conditions and equal inoculum. This information is valuable to industry stakeholders and growers, particularly basil growers with limited greenhouse or field space, and organic growers who primarily rely on resistant cultivars and cultural control methods to reduce disease pressure.
2.2 Materials and Methods

*Basil propagation*

Basil cultivar seeds were sown into soilless growing media (Promix BX Premier Horticulture Ltd., Qc) in 128 cell standard plug flats and thinned to one seedling per plug after 1-2 weeks. Individual plants were transplanted into 4” pots between 4-6 weeks after propagation. Potted basil plants were maintained in controlled-environment greenhouses set to 24°C (75°F) daytime and nighttime temperatures, with 14-10 hour day-night cycles, aided by supplemental lighting of half metal halide and half high-pressure sodium lights. Plants were regularly hand-watered and fertilized 3 times weekly during irrigation with Peters Professional 20-10-20 peat lite fertilizer at 200 ppm nitrogen.

*Peronospora belbahrii inoculation protocol*

Diseased basil plants were collected from garden centers, production fields and greenhouses, either as whole plants or cut stems. Sporulation was forced by incubating in a high humidity chamber (RH ≥96%) on a greenhouse bench overnight (≥12 hours) with 10 hours of darkness to ensure sporulation (Cohen et al., 2017). Sporulating leaves were collected and placed in sterile water, vortexed, and then filtered through a double layer of cheesecloth to remove debris. The sporangial suspension was then re-filtered through quadruple-layered cheesecloth to further remove sporangiophores, soil particles, and other debris. The double-filtered sporangia suspension was pelleted by centrifugation at 4000rpm for two minutes, the supernatant poured off, and then resuspended in sterile distilled water (diH₂O) and the centrifugation process was repeated for a second wash and pelleting. The washed pelleted sporangia were then resuspended in sterile diH₂O, and the suspension was quantified using a hemocytometer. Sporangia suspensions of 1x10⁶
sporangia/mL were aliquoted, pelleted and flash-frozen in liquid nitrogen and stored at -80°C for future DNA extraction. Sporangia suspensions of 1x10^4 sporangia/mL were prepared for inoculation of 6- to 8-week-old plants by spraying until run-off and incubating in a humidity chamber for 24 hours on the greenhouse bench. Only one isolate was maintained in a greenhouse at a time, if other isolates were collected, they were contained in different greenhouse rooms or growth chamber and sporulation was forced for a single isolate on any given day to reduce the possibility of cross-contamination.

P. belbahrii inoculum long-term survival assay

For long-term storage, *P. belbahrii* sporangia suspensions were prepared as described above, adjusted to 1x10^4 sporangia/mL and separated into 50 mL aliquots. These aliquots were vacuum filtered onto 55 mm sterile filter discs with 8 µm porosity to remove them from suspension, which was determined to cause no reduction in germination in preliminary trials (Supplementary Table 2.1). Two temperatures were chosen to test sporangia survivability: 20 and 25°C, representing optimal temperatures for *P. belbahrii* infection and average daily temperatures in a greenhouse climate during the Northeastern winter months. Two humidity treatments were included in this study, with saturated salt solutions of potassium chloride and potassium sulfate mixed to retain 84% and 96.5% RH *in vitro*, respectively (Winston & Bates, 1960). Platforms were constructed using 23-guage galvanized hardware cloth and 16-guage wire to fit 100 X 80 mm Pyrex crystallizing dishes (Supplementary Figure 2.1A). The filter discs were placed on the wire mesh platforms and positioned in Pyrex crystallizing dishes containing 25mL of saturated salt solution and sealed using 2 layers of parafilm. The salt solution treatments were randomly assigned time and temperature and placed accordingly into incubation.
chambers.

**In vitro germination assessment**

Preliminary trials of the *in vitro* germination assay revealed that *P. belbahrii* sporangia require a gradual rehydration period prior to re-suspension in water (as described in sporangia germination studies of *Phytophthora infestans* (Minogue & Fry, 1981)), and germination recovered after rehydration in a humidity chamber stored in a 10°C incubator for 2 hours (Supplementary Table 2). Following rehydration, stored filter discs were suspended in 50mL sterile water, vortexed, and used for *in vitro* germination and plant inoculations. One hundred microliters of the 1x10^4 sporangia/mL sporangial suspensions were plated onto 1.5% water agar in triplicate and spread using a sterile bent glass rod. The plates were then stored in a 10°C incubation chamber for 24 hours. Germination was assessed by examining the agar plates under a dissecting microscope and rating the presence of germ tubes (Supplementary Figure 1B) on 100 random sporangia per plate.

**Plant inoculation bioassay**

Re-hydrated 1x10^4 sporangia/mL suspensions were used to inoculate 8-week-old basil plants in triplicate per temperature and humidity treatment. Plants were spray inoculated using a handheld sprayer until run-off and placed in humidity chambers for 48 hours. A set of plants was inoculated with sterile water as a negative control for each time-point to ensure that there was no cross-contamination of inoculum. Plants were returned to humidity chambers after 6 days to induce sporulation.
\textit{P. belbahrii} isolate collection

Cut stems or whole plants of symptomatic basil were submitted by collaborators, extension agents, growers, and home gardeners to build a diverse collection of \textit{P. belbahrii} isolates. Sporulation was forced overnight as previously described, and fresh sporangia were collected, aliquoted, and flash-frozen for DNA extraction as described above.

\textit{P. belbahrii} isolate maintenance

\textit{P. belbahrii} isolate KAMA-20 was collected as an environmentally active isolate on susceptible ‘Genovese’ (Lot 48104) sentinel plants in 2020 in Worcester, MA. It was propagated on sweet basil cultivar ‘Genovese’ (Lot 48104) every 3 to 4 weeks in the greenhouse using the methods described above. \textit{P. belbahrii} isolate LIHREC-21 was collected as an environmentally active isolate on basil cultivar ‘Passion’ in Long Island, NY and propagated on ‘Devotion’ and ‘Passion’ every 3 to 4 weeks in the greenhouse. In late Fall 2021, after the field growing season was concluded in Massachusetts, \textit{P. belbahrii} isolate HVO-21 was collected from ‘Prospera® DMR (PS5)’ cultivated in a greenhouse production system and propagated on ‘Prospera® DMR (PS5)’ every 3 to 4 weeks in the greenhouse. The grower noted that several nearby fields of infected basil had been allowed to stay unharvested, allowing the pathogen to continue to propagate to the end of the season.

\textit{Basil cultivar trials}

A variety of downy mildew susceptible and resistant cultivars of basil were selected for greenhouse cultivar trials to establish differential virulence (further described

Evaluating basil downy mildew disease symptoms

Disease incidence (DI) was rated as a percentage of true leaves with symptoms and signs (interveinal chlorosis and/or visible sporulation) on affected plants (McGrath, 2020). The first six to eight true leaves (three to four leaf pairs) were rated, axillary leaves were not included as they had not emerged at the time of inoculation. Individual plants were assigned a disease severity (DS) score by examining the leaves assessed in the DI rating and assigning DS using an ordered categorical scale in which 0=no sporulation, 1=1-10%, 2=11-25%, 3=26-50%, 4=51-100% (Pyne et al., 2015).

Data Analysis

Analysis of variance (ANOVA) was performed to evaluate sporangia germination data and plant bioassay disease incidence data using the PROC GLM procedure in SAS Software v. 9.4 (SAS Institute Inc., Cary, NC, USA) and means were separated using Tukey’s HSD ($\alpha = 0.05$). Cultivar trial disease incidence data was analyzed by ANOVA and means were separated using Tukey’s HSD ($\alpha = 0.05$) in R (RStudio Team, 2020).
2.3 Results

*Establishment of Peronospora belbahrii inoculation protocol*

Many iterations of humidity chambers and sporangia handling procedures were tested to find a highly reproducible protocol resulting in consistent inoculation of multiple whole basil plants. Initially, the humidity chambers were constructed with a hole cut into the bins to allow a consumer-grade humidifier to be attached using consumer-grade pool tubing to direct humidity into the chambers (Figure 2.1A). Setting the humidifier to run for 30 minutes every 2 hours resulted in humidity readouts of 79.85% RH to 100% RH, with an average of 97.69% RH maintained over a 48-hour period, measured in January 2018 when temperatures ranged from 15.8°C to 24.1°C (Figure 2.1B). Later, the humidity chambers were altered to replace the humidifiers with 1-2 inches of standing water in the bottom (Figure 2.1C), which was sufficient to maintain high humidity. Over a 48-hour period in May 2022, the second generation humidity chambers maintained 86.62% RH to 98.92% RH with an average of 95.56% RH, at temperatures ranging from 20.0 to 36.9°C and an average temperature of 26.0°C (Figure 2.1D). These chambers provide adequate environmental conditions for *P. belbahrii* inoculation and incubation and can be disinfected easily after each round of experiments.
Figure 2.1. First and Second Generation High Humidity Chambers Used for Basil Downy Mildew Incubation and Inoculation. A. First generation high humidity chambers with commercial grade humidifiers. B. Temperature and relative humidity (RH) readouts from first generation humidifier, recorded in January 2018. C. Second generation high humidity chambers without additional humidifier. D. Temperature and relative humidity (RH) readouts from second generation humidifier, recorded in May 2022.

Survivability of *P. belbahrii* sporangia

The duration of survival of detached sporangia was assessed under two temperature treatments. 20 and 25°C, and two humidity levels, 84%RH and 96.5%RH, achieved by saturated salt solutions potassium chloride and potassium sulfate, respectively (Winston & Bates, 1960). The temperatures were chosen to represent the ideal temperature for *P. belbahrii* infection (20°C), and the average daily temperature of the greenhouses that we monitored (25°C). Ambient temperature and RH measured at canopy level in the same controlled-climate greenhouse in July 2017 had temperatures ranging from 20.9°C to 33.7°C, with an average temperature of 26.3°C. RH ranging from 61.20% to 89.94%, with an average ambient humidity of 75.74%. Ambient RH at the
canopy level in a high-production greenhouse can match or exceed these measurements for extended periods of time (as reported by one high-production grower), so long-term survivability of detached sporangia was measured at ideal temperatures of 20-25°C at RH≥84%.

The survivability of sporangia is anti-correlated with the storage time under both high (96.5%) and low (84%) relative humidity in two different temperatures (20 and 25°C) (Figure 2.2A, Supplementary Table 3). Humidity (84%RH vs. 86.5%RH) had significant effects on the rate of germination, while temperature was not a significant factor on in vitro germination (Supplementary Table 2). After 24 hours of storage time, the in vitro germination of detached P. belbahrii sporangia was reduced to ~86% across both temperature and RH levels. A clear reduction of the in vitro germination rates from 24 to 48 hours resulted in significantly different germination rates (P<0.05) of ~61.5% at 96.5% RH and ~17.5% at 84% RH. Detached P. belbahrii sporangia remained viable at 96.5%RH at a germination rate of ~40.4% after 144 hours at both temperatures, while viability was nullified by 120 hours in 84% RH regardless the temperature conditions (Figure 2.2A).

Along with the in vitro germination study, the in vivo plant infection bioassay was used to determine viability and infection potential following spore detachment and isolation. Similar to in vitro germination, the survivability of sporangia measured by disease incidence is anti-correlated with the storage time (Figure 2.2B). Disease incidence was measured as a percentage of infected leaves out of the first 6-8 true leaves rated (McGrath, 2020). We observed significant reductions in disease incidence after 24 hours at 25°C from ~54.77% to ~31.175% in both humidity treatments (Figure 2.2B)
(P<0.05). However, distinct from the in vitro germination assay, the overall effect of temperature (20 vs. 25°C) was significant on sporangia survival and infectivity (P<0.0001) (Supplementary Table 4). After 48 hpi, the disease incidences at 20°C maintained at ~45.06%, while the disease incidences at 25°C dropped to ~29.12% independent of RH levels (Figure 2.2B, Supplementary Table 5). The same trend was observed at 72 hpi, but by 120 hours the infectivity of sporangia was abolished for 84% RH at both temperatures, while sporangia stored at 96.5% RH at 20 and 25°C remained infective after 144 hours (Figure 2.2B).

Figure 2.2 Survival and infectivity of detached P. belbahrii sporangia. A. In vitro germination of P. belbahrii sporangia stored at 20°C (left) and 25°C (right) at 84% (blue) and 96.5% (purple) relative humidity (RH) treatments over 144 hours. B. Plant inoculation bioassay results plotted by disease incidence of basil inoculated with stored P. belbahrii sporangia at 20°C (left) and 25°C (right) at 84% (blue) and 96.5% (purple) relative humidity (RH) treatments over 144 hours.
Based on our data, high RH at temperatures of \( \leq 20^\circ C \) is a conducive environment for extended survival of detached \( P. \ belbahrii \) sporangia. To further define this survival interval, we performed an additional experiment, extending the length of storage time at 20°C in 96.5% RH. Survival of sporangia was reduced from 92% to 51.3% after 168 hours (7 days), and further reduced to 3.2% germination at 240 hours (10 days) (Figure 2.3A). Germination was abolished by 312 hours (13 days). Disease incidence followed a similar trend, with high levels of infection on all three inoculated plants per storage time treatment up to 168 hours (7 days), and reduced disease incidence levels after 192 hours (8 days) (Figure 2.3B). Sporangia infectivity was abolished at 288 hours (12 days). While germination could be recovered at 288 hours, the germination rate was 0.22%, suggesting that infection establishment may require a higher percentage of germinating sporangia (Figure 2.3).
Figure 2.3 Maximum survival and infectivity of *P. belbahrii* sporangia at 20°C and 96.5% RH. A. *in vitro* germination of *P. belbahrii* sporangia stored at 20°C and 96.5% RH over 360 hours. B. Plant inoculation bioassay results plotted by disease incidence of basil inoculated with stored *P. belbahrii* sporangia at 20°C and 96.5% RH over 360 hours.

*P. belbahrii* isolate collection

To assess the diversity of pathogen responsible for BDM, twenty-six unique *P. belbahrii* isolates were collected from infected basil plants from Florida, Kansas, Maine, Massachusetts, New Jersey, and New York from 2018-2022 (Table 2.2, Supplementary Figure 2). Sporangia samples were collected from whole plants or cut stems sporulated for ≥12 hours at high RH and filtered and washed as previously described. Of 26 samples, 16 isolates were retrieved from commercially available downy mildew resistant
<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Cultivar</th>
<th>Location</th>
<th>Date Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMASS</td>
<td>Genovese</td>
<td>Northampton, MA</td>
<td>2016</td>
</tr>
<tr>
<td>JRF-18</td>
<td>Prospera® DMR (PS5)</td>
<td>Albion, Maine</td>
<td>9/18/2018</td>
</tr>
<tr>
<td>LIHREC-18</td>
<td>Unknown</td>
<td>Long Island, NY</td>
<td>9/30/2018</td>
</tr>
<tr>
<td>EREC-18</td>
<td>Unknown</td>
<td>Florida</td>
<td>10/29/2018</td>
</tr>
<tr>
<td>HVO-18</td>
<td>Aroma 2</td>
<td>South Deerfield, MA</td>
<td>11/20/2018</td>
</tr>
<tr>
<td>LINY-19</td>
<td>Amazel</td>
<td>Long Island, NY</td>
<td>6/24/2019</td>
</tr>
<tr>
<td>NbMA-19</td>
<td>Genovese</td>
<td>Northborough, MA</td>
<td>7/26/2019</td>
</tr>
<tr>
<td>LwKS-19</td>
<td>Unknown</td>
<td>Lawrence, KS</td>
<td>7/31/2019</td>
</tr>
<tr>
<td>AmMA-LB-19</td>
<td>Lemon basil</td>
<td>Amherst, MA</td>
<td>9/11/2019</td>
</tr>
<tr>
<td>MMNY-19</td>
<td>Prospera® DMR (PS5)</td>
<td>Long Island, NY</td>
<td>9/18/2019</td>
</tr>
<tr>
<td>MMNY2-19</td>
<td>Thunderstruck</td>
<td>Long Island, NY</td>
<td>9/30/2019</td>
</tr>
<tr>
<td>HVO-19</td>
<td>Aroma 2</td>
<td>South Deerfield, MA</td>
<td>10/23/2019</td>
</tr>
<tr>
<td>KAMA-20</td>
<td>Genovese</td>
<td>Worcester, MA</td>
<td>9/7/2020</td>
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<td>AmNY-20</td>
<td>Amazel</td>
<td>Long Island, NY</td>
<td>10/14/2020</td>
</tr>
<tr>
<td>ObNY-20</td>
<td>Obsession</td>
<td>Long Island, NY</td>
<td>10/14/2020</td>
</tr>
<tr>
<td>PrNY-20</td>
<td>Prospera® DMR (PS5)</td>
<td>Long Island, NY</td>
<td>10/14/2020</td>
</tr>
<tr>
<td>HVO-20</td>
<td>Devotion</td>
<td>South Deerfield, MA</td>
<td>10/21/2020</td>
</tr>
<tr>
<td>PrILL2FL-21</td>
<td>Prospera® Italian Large Leaf DMR (ILL2)</td>
<td>Clewiston, FL</td>
<td>4/28/2021</td>
</tr>
<tr>
<td>HGCMA-21</td>
<td>Genovese, Sweet Bush, Sal's Italian Choice</td>
<td>Hadley, MA</td>
<td>6/17/2021</td>
</tr>
<tr>
<td>CCMA-21</td>
<td>Devotion and Obsession</td>
<td>Dennisport, MA</td>
<td>8/6/2021</td>
</tr>
<tr>
<td>BUNY-21</td>
<td>Obsession</td>
<td>Buffalo, NY</td>
<td>8/26/2021</td>
</tr>
<tr>
<td>LIHREC-21</td>
<td>Passion</td>
<td>Long Island, NY</td>
<td>9/15/2021</td>
</tr>
<tr>
<td>HFNJ-21</td>
<td>Rutgers DMR- unspecified</td>
<td>Hart Farm</td>
<td></td>
</tr>
<tr>
<td>RUSRF-21</td>
<td>Rutgers DMR- unspecified</td>
<td>Franklin Township, NJ</td>
<td></td>
</tr>
<tr>
<td>HVO-21</td>
<td>Prospera® DMR (PS5)</td>
<td>South Deerfield, MA</td>
<td>10/30/2021</td>
</tr>
<tr>
<td>AFFL-22</td>
<td>Ocimum tenuiflorum (Oci 145); Prospera® Italian Large Leaf DMR (ILL2); Dark Opal; O. selloi Green Pepper basil</td>
<td>Boynton Beach, FL</td>
<td>02/01/2022</td>
</tr>
</tbody>
</table>
Detection of *P. belbahrii* differential virulence pathotypes

Controlled-environment inoculations were performed to identify differential patterns of *P. belbahrii* virulence on a set of basil cultivars with different resistance classifications (Supplementary Table 6).

**Isolate KAMA-20 pathogenicity was restricted by available disease resistant cultivars, establishing the “baseline” virulence pathotype.**

Isolate KAMA-20 was collected from susceptible ‘Genovese’ sentinel plants in 2020. KAMA-20 was virulent on all susceptible cultivars tested, specifically ‘Genovese’, ‘diGenova’, and ‘Newton’, with average disease incidence ratings showing 100%, 100%, and 91.67% leaves infected, respectively. All three susceptible cultivars had disease severity ratings of 4, indicating that sporulation covered 51-100% of the leaf area (Figure 2.4, Table 2.2).

The intermediately resistant cultivar ‘Eleanora’ showed partial resistance, with average disease incidence ratings of 54.17%, but disease severity scores were 1 (1-10% leaf area covered) across all replicates and sporulation was limited.

Cultivar ‘Mrihani’ was highly resistant to the strain KAMA-20. Two plant replicates were healthy with no sign of infection, and one of three replicates had minor sporulation limited to nearly imperceptible areas of the ruffled leaf edges, resulting in an overall disease incidence score of 11.11% and a disease incidence rating of 1. Quantitatively resistant cultivars ‘Devotion’, ‘Obsession’, ‘Passion’, and ‘Thunderstruck’, progeny of the cross between ‘Mrihani’ and ‘Newton’, all showed total resistance to KAMA-20, with DS and DI scores of 0.
Qualitatively resistant cultivar ‘Prospera’ demonstrated total resistance and had DS and DI scores of 0 (Figure 2.4, Table 2.2).

All control plants of each cultivar, inoculated with sterile distilled water and incubated in an identical but separate humidity chamber on the same greenhouse bench, remained uninfected, indicating no prior inoculation or cross-contamination.

**Figure 2.4. Cultivar trial results for *P. belbahrii* isolate KAMA-20.** Cultivars are listed on the x-axis and grouped and colored by resistance classification (susceptible cultivars in green, intermediately resistant cultivar in pink, cultivars with quantitative resistance in blue, and cultivars with qualitative resistance in orange). Bars represent the mean disease incidence rating across three replicates per cultivar as a percentage of sporulating leaves per plant, and the error bars represent the standard deviation.

An additional assessment of KAMA-20 isolate disease incidence and severity was performed at 22 days post infection to identify any potential infection that was undetected
at 7dpi. All infected plants at 7dpi showed equal or greater signs and symptoms of infection at 22dpi (Table 2.2).

Susceptible ‘Genovese’, ‘diGenova’, and ‘Newton’, which exhibited serious disease 7 dpi, all retained the same disease incidence and severity scores. Intermediately resistant ‘Eleanora’ showed increased signs and symptoms of infection, with disease incidence of 66.67% and severity increasing to 3, indicating 26-50% of leaf area showing sporulation (Table 2.2). Notably, all three ‘Mrihani’ replicates had detectable but minimal sporulation on ruffled leaf edges and small patches of interveinal chlorosis, with a disease incidence score of 38.89% and disease severity remaining at 1. This could have been the result of secondary infection after the first assessment, undetectable infection on first assessment, or more likely a combination of both. Resistant ‘Devotion’, ‘Obsession’, ‘Passion’, ‘Thunderstruck’, and ‘Prospera’ all remained uninfected. One control ‘diGenova’ plant showed minimal sporulation, likely as a result of secondary infection and cross-contamination after the 7dpi assessment.
**Table 2.2 Results of KAMA-20 cultivar trial rated at 7- and 22-days post-infection**

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>7dpi Disease Incidence (%)</th>
<th>7dpi Disease Severity</th>
<th>22 dpi Disease Incidence (%)</th>
<th>22 dpi Disease Severity</th>
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</thead>
<tbody>
<tr>
<td>‘Genovese’-48104 (susceptible)</td>
<td>100</td>
<td>4</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>‘diGenova’ (susceptible)</td>
<td>100</td>
<td>4</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>‘Newton’ (susceptible)</td>
<td>92</td>
<td>4</td>
<td>92</td>
<td>4</td>
</tr>
<tr>
<td>‘Eleanora’ (intermediate resistance)</td>
<td>54</td>
<td>1</td>
<td>67</td>
<td>3</td>
</tr>
<tr>
<td>‘Mrihani’ (quantitative resistance)</td>
<td>11</td>
<td>1</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>‘Devotion’ (quantitative resistance)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Obsession’ (quantitative resistance)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Passion’ (quantitative resistance)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>‘Thunderstruck’ (quantitative resistance)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prospera® DMR (PS5) (qualitative resistance)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Isolate LIHREC-21 overcomes cultivars with quantitative resistance**

Isolate LIHREC-21 was collected from Rutgers DMR cultivars ‘Devotion’ and ‘Passion’ in a field cultivar trial in Long Island, New York in 2021. Notably, ‘Prospera’ grown in the same field trial remained uninfected (Figure 2.5).
Figure 2.5 Basil cultivar field trial identified isolate LIHREC-21 with differential virulence pathotype. ‘Prospera’ (left) is uninfected while growing side by side with Rutgers DMR ‘Devotion’ (right), which displays significant symptoms of infection. Photo provided by Dr. Margaret McGrath.

To further evaluate isolate LIHREC-21, greenhouse cultivar trials were performed as described above for KAMA-20. Intermediately resistant cultivar ‘Eleanora’ was omitted from this trial, and resistant cultivar ‘Prospera ILL’, sharing the hybrid source of resistance with all other ‘Prospera’ lines was added. Isolate LIHREC-21 was tested on susceptible ‘Genovese’ (lot 48104), ‘diGenova’, and ‘Newton, quantitatively-resistant ‘Mrihani’ and progeny ‘Devotion’, ‘Obsession’, ‘Passion’ and ‘Thunderstruck’, and qualitatively resistant ‘Prospera PS5’ and ‘Prospera ILL’.

At 7 dpi, all susceptible cultivars showed signs of heavy infection (Figure 2.6). ‘Genovese’ (lot 48104) had an average disease incidence score of 95.83%, and a DS of 4. Cultivars ‘diGenova’ and ‘Newton’ had DI scores of 100% and DS of 4 (Table 2.3).
Quantitatively resistant cultivars ‘Mrihani’, ‘Devotion’, ‘Obsession’, ‘Passion’, and ‘Thunderstruck’ were also heavily infected at 7dpi, with DI scores ranging from 75-100% and DS scores of 3-4 (Figure 2.6, Table 2.3).

Qualitatively resistant cultivars ‘Prospera’ and ‘Prospera ILL’, which derive their resistance from the nonhost Pb1 resistance gene introduced through interspecific hybridization (Ben-Naim et al., 2018; Ben-Naim & Weitman, 2021), remained completely resistant against LIHREC-21 (Figure 2.6, Table 2.3). All water-inoculated control plants remained uninfected.

This greenhouse trial confirmed the distinct disease phenotype of this new isolate, complementing the results of the field trial. The underlying genetic composition and mechanism for this virulence pathotype remain to be elucidated.
Figure 2.6 Cultivar trial results for *P. belbahrii* isolate LIHREC-21. Cultivars are listed on the x-axis and grouped and colored by resistance classification (susceptible cultivars in green, cultivars with quantitative resistance in blue, and cultivars with qualitative resistance in orange). Bars represent the mean disease incidence rating across three replicates per cultivar as a percentage of sporulating leaves per plant, and the error bars represent the standard deviation.

Isolate HVO-21 is highly virulent on ‘Prospera’, but has differential virulence on cultivars with quantitative resistance

Isolate HVO-21 was collected from ‘Prospera® DMR (PS5)’ grown in a commercial greenhouse in Whately, MA in the Fall of 2021.

Susceptible ‘Genovese’ (Lot 48104) and ‘Elidia’ inoculated with HVO-21 developed high levels of disease incidence (83% and 100%, respectively), and severity ratings of 2 at 7dpi (Figure 2.7, Table 2.3). Interestingly, ‘Mrhiani’ had the lowest level of disease incidence (28%) and severity (1) in this trial, suggesting that one or more sources of the quantitative resistance derived from this cultivar were more effective
against this isolate. This is further confirmed by the varying performance of ‘Mrihani’-derived cultivars, with ‘Devotion’, ‘Obsession’, and ‘Thunderstruck’ showing high susceptibility (85-100% incidence and severity scored of 2), but ‘Passion’ demonstrated improved resistance with a DI of 39% and DS rating of 1 (Figure 2.7, Table 2.3). All three of the ‘Prospera’ cultivars showed 100% disease incidence and relatively high disease severity scores of 3, as did the experimental line Genesis 164, demonstrating the ability of isolate HVO-2 to overcome these sources of resistance (Figure 2.7, Table 2.3).

Figure 2.7 Cultivar trial results for *P. belbahrii* isolate HVO-21. Cultivars are listed on the x-axis and grouped and colored by resistance classification (susceptible cultivars in green, cultivars with quantitative resistance in blue, and cultivars with qualitative resistance in orange). Bars represent the mean disease incidence rating across three replicates per cultivar as a percentage of sporulating leaves per plant, and the error bars represent the standard deviation.
The overall results show differential virulence phenotypes among all three isolates (Table 2.3). While not every cultivar was tested against every isolate, each differential panel was effective to determine isolate virulence across the available sources of resistance. The results of these differential panels suggest the emergence of new pathotypes of *P. belbahrii* capable of overcoming resistance.
Table 2.3 Cultivar trial results of all tested *P. belbahrii* isolates

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Resistance classification</th>
<th>KAMA-20 Disease Incidence (%), Severity</th>
<th>LIHREC-21 Disease Incidence (%), Severity</th>
<th>HVO-21 Disease Incidence (%), Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Genovese’ Lot 48104</td>
<td>Susceptible</td>
<td>100, 4 a</td>
<td>96, 4 a</td>
<td>100, 4 a</td>
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<tr>
<td>‘diGenova’</td>
<td>Susceptible</td>
<td>100, 4 a</td>
<td>100, 4 a</td>
<td>-</td>
</tr>
<tr>
<td>‘Genovese’</td>
<td>Susceptible</td>
<td>-</td>
<td>-</td>
<td>83, 2 a</td>
</tr>
<tr>
<td>‘Elidia’</td>
<td>Susceptible</td>
<td>-</td>
<td>-</td>
<td>100, 2 a</td>
</tr>
<tr>
<td>‘Newton’</td>
<td>Susceptible</td>
<td>92, 4 a</td>
<td>100, 4 a</td>
<td>100, 4 a</td>
</tr>
<tr>
<td>‘Eleanora’</td>
<td>Intermediate Resistance</td>
<td>54, 1 b</td>
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<td>-</td>
</tr>
<tr>
<td>‘Mrihani’</td>
<td>Resistant-quantitative</td>
<td>11, 1 c</td>
<td>75, 3 a</td>
<td>28, 1 b</td>
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<td>‘Devotion’</td>
<td>Resistant-quantitative</td>
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<td>100, 3 a</td>
<td>100, 2 a</td>
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<td>‘Obsession’</td>
<td>Resistant-quantitative</td>
<td>0, 0 c</td>
<td>92, 4 a</td>
<td>100, 2 a</td>
</tr>
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<td>Resistant-quantitative</td>
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<td>96, 3 a</td>
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<td>‘Thunderstruck’</td>
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</tr>
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<td>Prospera® PL4, F1 (Potted Large-Leaf DMR)</td>
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<td>-</td>
<td>-</td>
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<td>Genesis 164</td>
<td>Resistant-major</td>
<td>-</td>
<td>-</td>
<td>100, 3 a</td>
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</table>
2.4 Discussion

The practice of maintaining an active isolate in a closed and controlled environment has advantages for continued experimental study. However, obligate pathogens are notoriously difficult to study due to their recalcitrance to axenic culturing. This research has resulted in the development, refinement, and utilization of replicable protocols for collecting and maintaining \( P. \) \( belbahrii \) isolates for large-scale studies. The inoculation protocol has been utilized for greenhouse cultivar trials, \( P. \) \( belbahrii \) isolate maintenance, and inoculation for DNA and RNA sample collections, and can be found in APPENDIX 2. The major advantages of the protocol are the ease of use and the large amount of \( P. \) \( belbahrii \) inoculum that can be collected and stored, as well as the use of inexpensive, consumer-grade materials that can be used in low resource settings and can be easily disinfected or replaced. Since beginning this research, there have been new studies that describe similar methods, in particular the maintenance of a \( P. \) \( belbahrii \) isolate on susceptible plants using a variety of high humidity or dew chambers (Cohen & Rubin, 2015; Cohen & Ben-Naim, 2016; Shao & Tian, 2018). To our knowledge, we are the only group that is maintaining multiple \( P. \) \( belbahrii \) isolates on resistant cultivars.

The studies of long-term sporangia survival align well with the results previously described, showing maximal survival and infectivity of up to 96 hours at RH levels of 84% (Cohen & Ben-Naim, 2016; Cohen et al., 2017). While the higher temperature of 25°C reduced germination compared to 20°C, temperature was not found to be a significant factor for survival, although it was significant for infectivity. Other studies have demonstrated that temperatures above 25°C will impact \( P. \) \( belbahrii \) survival (Cohen & Rubin, 2015; Cohen et al., 2017). Much longer survival periods than have
previously been reported for detached sporangia, up to 264 hours, were observed at 20°C and 96.5% RH compared to 96-120 hours at lower humidity and higher temperatures. Overall, our data suggests that RH is a significant factor in sporangia longevity, and infectivity was extended at the lower temperature and higher humidity treatments. Further, we found that low levels of germination could still result in significant infections. This is important to inform recommendations to greenhouse growers who have faced an outbreak of basil downy mildew; sporulating plant material should be destroyed, and the space should be vacant at least 5 days prior to re-establishing basil crops in the area. Additionally, raising temperatures and taking measures to reduce humidity will decrease the survival of *P. belbahrii* inoculum, and may be used to shorten this safe re-entry interval. If all plant material is not removed, the potential for continued infection increases due to the polycyclic nature of basil downy mildew and the long period of latent infection before sporulation,

The differential *P. belbahrii* isolate virulence and cultivar performance studies confirmed the presence of multiple pathotypes of basil downy mildew in North America. These greenhouse trials served to complement and confirm results from various field trials, which have shown that, in some years and locations, different resistant cultivars have become more heavily infected by BDM (Patel *et al.*, 2021; McGrath, 2022). The occurrence of *P. belbahrii* pathotypes/races 0 and 1 have been reported as of 2021, with race 0 defined by avirulence on ‘Prospera’ cultivars controlled by resistance gene Pb1 and a unique SSR profile (Ben-Naim & Weitman, 2021). Race 1 was defined by virulence of an isolate on ‘Prospera’ cultivars originally reported in New Jersey, with additional confirmation of a unique SSR profile (Ben-Naim & Weitman, 2021), and our
observations support the occurrence of this pathotype/race in multiple years and locations in the US (Table 2). The cultivar trial results indicate the isolate KAMA-20 belongs to *P. belbahrii* pathotype/race 0, with avirulence on ‘Prospera’ cultivars as well resistant Rutgers DMRs. Of interest in the KAMA-20 trials is the result of the Rutgers DMR lines demonstrating improved resistance compared to their resistant breeding grandparent ‘Mrihani’. This could suggest that the inbreeding and crossing process strengthened and/or fixed the effects of ‘Mrihani’ derived quantitative resistance in the progeny.

Isolate LIHREC-21 was also avirulent on ‘Prospera’, but significantly virulent on the Rutgers DMR cultivars, indicating that it may belong to a unique pathotype. The Rutgers DMR cultivars have quantitative resistance, and there is not a clear delineation of pathogen pathotype as defined by classical major gene resistance, however our results show a clear and distinct difference between the virulence of KAMA-20 (pathotype 0) and LIHREC-21.

By contrast, HVO-21 could belong to pathotype 1, though its demonstrated virulence on breeding line Genesis 164 may indicate that it has a unique ability to overcome Pb1 and a new source of resistance, not previously reported in the Israeli study (Ben-Naim & Weitman, 2021). The reduced virulence of HVO-21 on ‘Mrihani’ and ‘Passion’ is intriguing and demonstrates the utility of quantitative resistance sources. Ideally, combining these sources of resistance in the future will create more durable and stable cultivars.

To our knowledge, this study is the first to test multiple *P. belbahrii* isolates against cultivar panels under controlled conditions. As possible new BDM pathotypes emerge and new BDM resistant sweet basil lines are developed, these cultivar trials will
need to be replicated additional cultivars tested and complemented with field trials in the future.
2.5 References


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Supplementary Table 1. Assessment of vacuum-filtration impact on *P. belbahrii* sporangia germination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germination of <em>P. belbahrii</em> sporangia in 1.5% water agar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plate 1</td>
</tr>
<tr>
<td>Fresh sporangia, unfiltered</td>
<td>77</td>
</tr>
<tr>
<td>Fresh sporangia, filtered and resuspended, Replication 1</td>
<td>84</td>
</tr>
<tr>
<td>Fresh sporangia, filtered and resuspended, Replication 2</td>
<td>86</td>
</tr>
<tr>
<td>Fresh sporangia, filtered and resuspended, Replication 3</td>
<td>78</td>
</tr>
</tbody>
</table>
Supplementary Table 2. ANOVA Table for *in vitro* germination

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage Time (Days)</td>
<td>6</td>
<td>69740</td>
<td>11623</td>
<td>777.116</td>
<td>2e-16 ***</td>
</tr>
<tr>
<td>Humidity</td>
<td>1</td>
<td>27807</td>
<td>27807</td>
<td>1859.095</td>
<td>&lt; 2e-16 ***</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>29</td>
<td>29</td>
<td>1.950</td>
<td>0.16814</td>
</tr>
<tr>
<td>Days*Humidity</td>
<td>6</td>
<td>11498</td>
<td>1916</td>
<td>128.125</td>
<td>&lt; 2e-16 ***</td>
</tr>
<tr>
<td>Days*Temperature</td>
<td>6</td>
<td>202</td>
<td>34</td>
<td>2.249</td>
<td>0.05156</td>
</tr>
<tr>
<td>Humidity*Temperature</td>
<td>1</td>
<td>124</td>
<td>124</td>
<td>8.282</td>
<td>0.00566 **</td>
</tr>
<tr>
<td>Days<em>Humidity</em>Temperature</td>
<td>6</td>
<td>925</td>
<td>154</td>
<td>10.302</td>
<td>1.15e-07 ***</td>
</tr>
<tr>
<td>Residuals</td>
<td>56</td>
<td>838</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Supplementary Table 3. *in vitro* germination rate means of detached *P. belbahrii* sporangia under controlled temperature and humidity treatments over time.

The four temperature x humidity treatments are listed across the top row of the table, and the hours of storage of detached sporangia is listed in the first column. Average means of three replicates were assessed using ANOVA. Different lowercase letters indicate statistical differences across temperature and humidity treatments at each timepoint based on Tukey's 'Honest Significant Difference' method (*p* ≤ 0.05).

<table>
<thead>
<tr>
<th>Storage Time (hours)</th>
<th>84% RH, 20°C</th>
<th>84% RH, 25°C</th>
<th>96.5% RH, 20°C</th>
<th>96.5% RH, 25°C</th>
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<tr>
<td>0</td>
<td>97.17 a</td>
<td>97.17 a</td>
<td>97.17 a</td>
<td>97.17 a</td>
</tr>
<tr>
<td>24</td>
<td>85.95 ab</td>
<td>82.33 bc</td>
<td>90.10 ab</td>
<td>85.67 ab</td>
</tr>
<tr>
<td>48</td>
<td>22.67 hi</td>
<td>12.33 ij</td>
<td>52.33 ef</td>
<td>70.67 cd</td>
</tr>
<tr>
<td>72</td>
<td>10.33 ij</td>
<td>0.67 j</td>
<td>61.00 de</td>
<td>73.00 cd</td>
</tr>
<tr>
<td>96</td>
<td>1.67 j</td>
<td>0 j</td>
<td>67.20 d</td>
<td>67.51 de</td>
</tr>
<tr>
<td>120</td>
<td>0 j</td>
<td>0 j</td>
<td>44.09 fg</td>
<td>44.17 fg</td>
</tr>
<tr>
<td>144</td>
<td>0 j</td>
<td>0 j</td>
<td>48.03 fg</td>
<td>32.76 gh</td>
</tr>
</tbody>
</table>
Supplementary Table 4. ANOVA Table for plant bioassay

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>MS</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage Time (Days)</td>
<td>6</td>
<td>26397</td>
<td>4400</td>
<td>108.741</td>
<td>&lt; 2e-16 ***</td>
</tr>
<tr>
<td>Humidity</td>
<td>1</td>
<td>638</td>
<td>638</td>
<td>15.762</td>
<td>0.000207 ***</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>2947</td>
<td>2947</td>
<td>72.830</td>
<td>1.03e-11 ***</td>
</tr>
<tr>
<td>Days*Humidity</td>
<td>6</td>
<td>1035</td>
<td>172</td>
<td>4.263</td>
<td>0.001322 **</td>
</tr>
<tr>
<td>Days*Temperature</td>
<td>6</td>
<td>1602</td>
<td>267</td>
<td>6.598</td>
<td>2.59e-05 ***</td>
</tr>
<tr>
<td>Humidity*Temperature</td>
<td>1</td>
<td>22</td>
<td>22</td>
<td>0.533</td>
<td>0.468202</td>
</tr>
<tr>
<td>Days<em>Humidity</em>Temperature</td>
<td>6</td>
<td>662</td>
<td>110</td>
<td>2.727</td>
<td>0.021445 *</td>
</tr>
<tr>
<td>Residuals</td>
<td>56</td>
<td>2266</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Supplementary Table 5. Disease Incidence means of plant bioassay results of *P. belbahrii* sporangia survival studies. The four temperature x humidity treatments are listed across the top row of the table, and the hours of storage of detached sporangia is listed in the first column. Average means of three replicates were assessed using ANOVA. Different lowercase letters indicate statistical differences across temperature and humidity treatments at each timepoint based on Tukey’s ‘Honest Significant Difference’ method ($p \leq 0.05$)

<table>
<thead>
<tr>
<th>Storage Time (hours)</th>
<th>84% RH, 20°C</th>
<th>84%RH, 25°C</th>
<th>96% RH, 20°C</th>
<th>96% RH, 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>54.77 a</td>
<td>54.77 a</td>
<td>54.77 a</td>
<td>54.77 a</td>
</tr>
<tr>
<td>24</td>
<td>52.44 ab</td>
<td>28.33 cde</td>
<td>36.87 abcd</td>
<td>34.02 bcd</td>
</tr>
<tr>
<td>48</td>
<td>45.52 abc</td>
<td>28.02 cdef</td>
<td>44.60 abc</td>
<td>30.22 cde</td>
</tr>
<tr>
<td>72</td>
<td>26.23 cdef</td>
<td>0 h</td>
<td>35.33 abcd</td>
<td>3.57 gh</td>
</tr>
<tr>
<td>96</td>
<td>7.98 fgh</td>
<td>0 h</td>
<td>19.91 defgh</td>
<td>3.04 gh</td>
</tr>
<tr>
<td>120</td>
<td>0 h</td>
<td>0 h</td>
<td>20.35 defg</td>
<td>3.33 gh</td>
</tr>
<tr>
<td>144</td>
<td>0 h</td>
<td>0 h</td>
<td>20.78 defg</td>
<td>13.65 efgh</td>
</tr>
</tbody>
</table>
Supplementary Table 6. Basil cultivars screened against *P. belbahrii* isolates. Details of cultivar resistance classifications, resistance sources, seed sources, and additional information is listed.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Resistance Classification</th>
<th>Source of Resistance</th>
<th>Source of Seed</th>
<th>Other Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Genovese’</td>
<td>Susceptible</td>
<td>N/A</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>‘Genovese’ Lot 48104</td>
<td>Susceptible</td>
<td>N/A</td>
<td>Johnny’s Selected Seeds</td>
<td>An older seed lot originally used for Fusarium trials and BDM propagation</td>
</tr>
<tr>
<td>‘diGenova’</td>
<td>Susceptible</td>
<td>N/A</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>‘Eliidia’</td>
<td>Susceptible</td>
<td>N/A</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>‘Newton’</td>
<td>Susceptible</td>
<td>N/A</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>‘Eleanora’</td>
<td>Intermediate Resistance</td>
<td>Unknown</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>‘Mrihani’</td>
<td>Quantitative Resistance</td>
<td></td>
<td>Strictly Medicinal Seeds</td>
<td></td>
</tr>
<tr>
<td>Rutgers ‘Devotion’ DMR</td>
<td>Quantitative Resistance</td>
<td>‘Mrihani’</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>Rutgers ‘Obsession’ DMR</td>
<td>Quantitative Resistance</td>
<td>‘Mrihani’</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>Rutgers ‘Passion’ DMR</td>
<td>Quantitative Resistance</td>
<td>‘Mrihani’</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>Rutgers ‘Thunderstruck’ DMR</td>
<td>Quantitative Resistance</td>
<td>‘Mrihani’</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>Prospera® PS5, F1 (Potted Small-Leaf DMR)</td>
<td>Qualitative Resistance</td>
<td><em>O. americanum</em> Pb1A and Pb1A’</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>Prospera® ILL2, F1 (Italian Large-Leaf DMR)</td>
<td>Qualitative Resistance</td>
<td><em>O. americanum</em> Pb1A and Pb1A’</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>Prospera® PL4, F1 (Potted Large-Leaf DMR)</td>
<td>Qualitative Resistance</td>
<td><em>O. americanum</em> Pb1A and Pb1A’</td>
<td>Johnny’s Selected Seeds</td>
<td></td>
</tr>
<tr>
<td>Genesis 164</td>
<td>Qualitative Resistance-stacked</td>
<td><em>O. americanum</em> Pb1A and Pb1A’ + ?</td>
<td>Collaborators</td>
<td>New breeding line with second major resistance gene</td>
</tr>
</tbody>
</table>
Supplementary Figure 1. *in vitro* sporangia storage and germination. **A.** Filter discs with back-filtered sporangia on wire mesh platforms placed in Pyrex crystallizing dishes containing 25mL saturated salt solution. **B.** *P. belhahri*i sporangia germinated on 1.5% water agar, visualized at 50X magnification.
Supplementary Figure 2. Infected plants collected for *P. belbahrii* isolate collection. A. Cut stems packaged and shipped. B. Upon receipt, cut stems were kept in water and BDM sporulation was forced overnight at high RH.
CHAPTER 3

IDENTIFICATION OF NOVEL BASIL DOWNY MILDEW RESISTANCE GENES USING DE NOVO COMPARATIVE TRANSCRIPTOMICS

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3.1 Introduction

Basil (genus *Ocimum*) is a major herb crop with diverse species and cultivars possessing distinct phenotypes in plant size, leaf shape, aroma, and flavor (Vieira & Simon, 2006). Sweet basil (*Ocimum basilicum* L.) is the most popular basil and is cultivated for culinary use and essential oil production for applications including medicine, health care products, and food additives. In 2019, revenue generated in the US from sweet basil and other culinary herbs grown for dry processing and fresh market sales was estimated to be $165 million dollars, and other estimates have even valued the retail market above $300 million dollars (Wyenandt *et al.*, 2015; (*dataset*) USDA National Agricultural Statistics Service (2017).

Basil Downy Mildew (BDM), caused by the biotrophic oomycete *Peronospora belbahrii*, has become the most important disease posing a serious threat to global basil production since its introduction and movement in Europe in 2001 and the US in 2007 (Belbahri *et al.*, 2005; Wyenandt *et al.*, 2015). The pathogen enters plant tissue through open stomata or direct penetration of the upper cuticle, and colonizes leaf tissue intercellularly, causing characteristic symptoms of interveinal chlorosis several days after infection (Wyenandt *et al.*, 2015; Cohen *et al.*, 2017). Following sustained periods of high relative humidity (>85%), the pathogen produces sporangiophores bearing infective sporangia, which emerge through stomata and create a gray to dark-gray discoloration corresponding to interveinal chlorosis (Wyenandt *et al.*, 2015; Cohen *et al.*, 2017). The sporangia are aerially dispersed and cause polycyclic infections throughout large areas of production (Wyenandt *et al.*, 2015; Cohen *et al.*, 2017). After prolonged infection, the leaves desiccate and are abscised from the plant (Wyenandt *et al.*, 2015; Cohen *et al.*, 2017).
Symptoms and signs of BDM disease render plants unfit for commercial sale (Wyenandt et al., 2015). First reported in Uganda in 1930, BDM began to attract attention in 2001 when disease instances were increasingly reported across the world with reports from the Americas, Asia, and Europe (Garibaldi et al., 2004, 2005; McLeod et al., 2006; Khateri et al., 2007; Roberts et al., 2009; Ronco et al., 2009; Martínez de la Parte et al., 2010; Nagy & Horváth, 2011; Kanetis et al., 2014; Šafránková & Holková, 2014; Choi et al., 2016). BDM was first reported in the US in 2007 in Florida, and then in the Northeast the following year (Roberts et al., 2009; Wyenandt et al., 2015). As of 2021, BDM has been reported in 44 US states, including Hawaii and the District of Columbia, threatening productivity in every growing region (Wyenandt et al., 2015; McGrath, 2021, 2022).

Chemical and cultural control methods to prevent infection and reduce disease spread include conventional fungicides (Homa et al., 2014; McGrath, 2020; Patel et al., 2021), nocturnal fanning (Cohen & Ben-Naim, 2016), nocturnal illumination (Cohen et al., 2013; Patel et al., 2021), and daytime solar heating (Cohen & Rubin, 2015). However, these measures can be prohibitively time consuming and costly, necessitating the development of improved basil cultivars with BDM resistance. Initial screening of basil germplasm identified the *O. basilicum* ‘Mrihani’ (MRI) (Horizon Seed Co., Williams, OR) with significant resistance to BDM (Pyne et al., 2015). This cultivar has a unique anise/fennel aroma and flavor profile, and a distinctive phenotype as compared to other *O. basilicum* cultivars selected for culinary use. For example, the MRI leaves are smaller than other basil cultivars and have serrated leaves compared to the large downward cupped smooth leaves of commercial sweet basil (Figure 3.1A). Most
importantly, the taste and smell of MRI due to its unique methyl chavicol chemotype differs considerably from eugenol-enriched sweet basil (Pyne et al., 2015). This cultivar was used in a parental cross with BDM-susceptible and the Fusarium-resistant ‘Newton’ (also referred to as Rutgers breeding line SB22), and successfully produced fertile offspring. From the six-generation breeding design, four BDM resistant cultivars (‘Devotion’, ‘Obsession’, ‘Passion’, and ‘Thunderstruck’) were selected from the backcrossed population progeny with improved downy mildew resistance and desirable phenotypes and chemotypes (Simon et al., 2018).

Screening of the full-sibling family offspring of the MRI x SB22 cross revealed additive and dominant gene effects of the MRI-conferred resistance, leading to the hypothesis that dominant alleles are involved in resistance (Pyne et al., 2015). A quantitative trait locus (QTL) analysis of the F2 mapping population from the cross between MRI and SB22 identified a single locus (dm11.1) that accounts for 20%-28% of the variance observed among the F2 population (Pyne et al., 2017). In addition, this study also identified two minor loci (dm9.1 and dm14.1) that respectively contributed 5-16% and 4-18% of the F2 population’s phenotypic variation. Resistance (R) genes involved in quantitative disease resistance map to QTLs (Nelson et al., 2018), and the results of the linkage mapping performed in the ‘MRI’ X SB22 F2 mapping population suggest that these loci may contain genes conferring quantitative disease resistance in ‘MRI’. Without genome assemblies of the basil cultivars, the underlying causative genes remained unknown.

A comparative transcriptomic analysis was designed to identify unique genes involved in the interactions of the resistant cultivar MRI and the susceptible commercial
cultivar SB22 with the pathogen *P. belbahrii*. RNA was extracted at 12, 24, 48, and 72 hours post inoculation (hpi) representing roughly germination, penetration, and intercellular growth stages. Global transcription expression profiles identified three categories of genes uniquely induced in the MRI cultivar upon pathogen challenge, including R genes encoding nucleotide-binding leucine rich repeat proteins (NLRs), receptor-like kinases (RLKs) that sense conserved microbe-associated molecular patterns, and secondary metabolic enzymes. Validation of the top candidate resistance NLR protein-encoding gene confirmed its unique presence in the MRI cultivar as well as two out of the four resistance hybrids. Unique upregulation of the salicylic acid synthesis pathway in MRI suggests the perturbation of this important hormone signaling pathway in conferring BDM resistance.

### 3.2 Materials and Methods

**Sample preparation**

Inbred *O. basilicum* genotypes SB22 (*P. belbahrii* susceptible) and MRI (*P. belbahrii* resistant) plants were grown from seed. Previously infected sweet basil leaves with fresh *P. belbahrii* sporulation were harvested and agitated for 5 minutes in sterile distilled water (diH$_2$O). The inoculum mixture was filtered with 40 μm nylon mesh. A 1 mL subsample from the filtered inoculum was pipetted into an Eppendorf tube and frozen at -80°C to serve as a pathogen control. The remaining inoculum was centrifuged at 1,000g for 1 min and diH$_2$O decanted. The resulting sporangia pellet was resuspended in diH$_2$O, and the inoculum concentration was adjusted to 1 x 10$^5$ sporangia/mL. Four-to-six-week-old MRI and SB22 plants were spray-inoculated at the 6-leaf (3 true leaf set) growth stage with approximately 1 mL/leaf and plants were incubated at 100% relative
humidity for 24 hours. A set of MRI and SB22 plants were sprayed with diH2O in triplicate to serve as the mock inoculated control.

Four disks per true leaf were sampled from both genotypes at 12, 24, 48 and 72 hpi and immediately flash frozen in liquid nitrogen. The water control leaves were harvested at 12 hpi only. Total RNA was extracted from freshly ground tissue using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich). RNA samples were used to generate sequence libraries using a library prep kit from New England Biolabs (NEB #E7530). Paired-end sequence reads of 75 bp were generated at the TUFTs genomic center at the Tufts University School of Medicine using the Hi-Seq Illumina platform. Higher coverage analyses were specifically designed for inoculated samples that contain both the pathogen and the host due to the increased complexity of these samples, allowing for further study of pathogen expression.

Generating transcript assemblies and FPKM expression

FASTQC version 0.11.5 (Andrews, 2010) was used to assess average read quality. Paired-end reads (fastq files) were provided to Trinity version 2.4.0 and assembled using default parameters (Grabherr et al., 2011). Datasets were assembled including single sets using either all MRI datasets and the sporangia control (MRI Combined Assembly) or all SB22 datasets including the sporangia control (SB22 Combined Assembly). Separately assembled control data provided organism specific databases of genes and transcripts.

The resulting output files served as the references for expression quantification. RSEM version 1.2.29 (Li & Dewey, 2011) and bowtie version 1.0.0 (Langmead et al., 2009) were used to calculate FPKM (Fragments per Kilobase exon per Million mapped.
reads) values for assembled contigs while tracking replicate information. RNAseq datasets from both MRI and SB22 were mapped to the infected MRI Combined Assembly to standardize the reference which allowed us to use previously generated gene annotations and to cluster genes from both cultivars together. In all cases the standard settings were used for assembly and transcript quantification. Additionally, edgeR (Robinson et al., 2010) was used to calculate differential gene expression. Expression data from both MRI and SB22 data mapped to the MRI Combined Assembly using Trinity and edgeR was used to assess differential expression between all timepoints within a single cultivar. Trinity DEG output data was filtered for genes with a p-value less than 0.05 and FDR less than 0.01.

Reciprocal BLAST hits and reference gene phylogeny

To explore the overall sequence conservation between MRI and SB22, we performed a reciprocal BLAST using all sequences within the MRI and SB22 water control assemblies. Briefly, all MRI sequences were compared to the SB22 transcriptome, and all SB22 sequences were compared to the MRI transcriptome. The hit with the highest BLAST score for each gene was chosen. Results were compared and pairs of top scoring genes were considered reciprocal best BLAST hits (i.e., MRI gene X BLASTs to SB22 gene Z, and SB22 gene Z BLASTs to MRI gene X).

Sequence conservation between MRI and SB22 was further assessed by performing a phylogenetic analysis using 9 protein-coding chloroplast genome genes based on a prior analysis (Rastogi et al., 2015). The MRI and SB22 chloroplast genes were identified using BLAST against the MRI and SB22 water control assemblies, and the top hits with the highest bit scores were chosen and translated into coding sequences.
Sequence alignment of the MRI and SB22 coding sequences was performed against sequences from 14 asterid lineage plants downloaded from NCBI Organelle Genome Resources database, with *Spinacia oleracea* L. and *Arabidopsis thaliana* L. set as outgroups (Rastogi et al., 2015). The gene sequences were aligned using MAFFT (Madeira et al., 2019), and the tree was generated using IQ-TREE (Minh et al., 2020).

**Sequence Translation and Annotation**

We generated a database of sequence annotations for MRI genes. All MRI genes with an expression of FPKM >1 in at least one time point were chosen and the longest transcript associated with that gene was compared to the NCBI non-redundant database using cloud BLAST through Blast2GO. Annotations were saved as a searchable database in text format. Genes were filtered by taxonomic hit to verify their species of origin as needed.

To facilitate easier searches for gene families of interest, we translated the longest nucleotide sequence associated with each gene into six-frame translated protein sequences using EMBOSS, searching for only those translated sequences between START and STOP codons longer than 30 amino acids. In many cases to verify protein domain structure, the nucleotide or protein sequence was analyzed using either the NCBI conserved domain finder (Marchler-Bauer et al., 2015), PFAM (Finn et al., 2016), or InterProScan (Jones et al., 2014). Sequences were aligned using MEGA 6 for visual inspection (Tamura et al., 2013).
Expression clustering toward candidate resistance gene identification

Genes were clustered using Trinity version 2.2.0 based on read counts following the steps outlined in the Trinity manual (Robinson et al., 2010). Expression data generated by mapping all datasets to the MRI Combined Assembly were used for clustering. A matrix of gene expression at all timepoints and replicates was used to define clusters with the edgeR function associated with Trinity, using \( p=50 \) and \( p=20 \) (a grouping parameter for cluster creation, with higher numbers forming larger and broader clusters). The resulting clusters, available in pdf format, were visually examined for clusters which displayed the target expression profile.

BLAST search for secondary metabolite enzyme and resistance genes

To analyze MRI unique gene families and defense hormone signaling genes, we performed BLASTp search against MRI and SB22 translated nucleotide sequences using *A. thaliana*, or in some cases sweet basil, protein sequences retrieved from NCBI. Generally, the hit with the highest bit score was chosen as the top hit for each sequence. In cases of short alignment length or low sequence identity, the recovered MRI or SB22 hit was compared to the green plant database on NCBI. BLAST version 2.2.22 was used in all cases to compare protein sequences (Altschul et al., 1990) at the Massachusetts green-energy high performance computing center (MGHPCC).

PCR screen of parent and cultivar genomic DNA for unique genes

Genomic DNA was prepared from approximately 80mg of newly emerging leaf tissue of MRI, SB22, ‘Devotion’, ‘Obsession’, ‘Passion’ and ‘Thunderstruck’ cultivars using the E.Z.N.A. SP Plant DNA Kit (Omega BioTek, Norcross, GA) (Pyne et al.,
2017). Primers amplifying transcript sequences were designed for MRI and SB22 shared genes as well as MRI unique genes including the comp160460c0 transcript, and were ordered from IDT (Coralville, IA). The primers were either external primers designed to amplify the whole gene (MRI_134-F 5’-CCGAGAAAATCGATCTAGAGAG-3’, MRI_2869-R 5’-CTAGCTTGATCTTTTAATTGGTGGAAAAAT-3’) or internal primers for specific regions of interest (Supporting Information Table S1). Primers amplifying a 198bp fragment of the *O. basilicum* Actin gene (ObActin_2-F 5’-GTTATGCACTTCCCCATGCT-3’, ObActin_2-R 5’-GAGCTGTTCTTTGCGGTCTC-3’) were used in positive control reactions for all cultivars. PCR was performed with Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) using manufacturer-recommended cycling conditions with a 30 second denaturation cycle to ensure full denaturation of genomic DNA, a 30 second extension time to amplify the 198bp ObActin region and a 120 second extension time to amplify the ~3.5kb comp160460-encoded gene on a Mastercycler proS (Eppendorf, Hamburg, Germany). Water was used as a negative control template in all reaction sets. Amplicons were visualized on 1.5% agarose gels stained with SYBR Safe DNA Gel Stain (Invitrogen, Waltham, MA), and imaged under UV light.

*NLR allele analysis*

Successfully amplified comp160460c0 products from MRI, ‘Devotion’ and ‘Obsession’ were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and single amplicon copies were ligated into the pMiniT 2.0 vector using the NEB PCR Cloning Kit (New England Biolabs, Ipswich, MA). Individual clones were selected and confirmed via colony PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA).
England Biolabs, Ipswich, MA) with primers 1BF and 8R which were designed to amplify the coiled coil and NB-ARC domain coding sequences of MRI-R1. Plasmid DNA was prepared from confirmed clones using the Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine, CA).

Individual comp160460c0 clones from MRI, ‘Devotion’ and ‘Obsession’ were sequenced using NEB PCR Cloning Kit Cloning Analysis Forward and Reverse primers to flank the insert, as well as internal primers (Supporting Information Table S1). Twelve clones were sequenced from MRI, and six clones were sequenced from both ‘Devotion’ and ‘Obsession’. Consensus sequences for each clone were assembled and annotated to identify intron regions by alignment to transcript sequences; coding regions were confirmed and annotated using InterProScan (Jones et al., 2014). Assembled sequences were identified as individual alleles, nucleotide and predicted protein sequences were aligned using the EMBL-EBI Clustal Omega multiple sequence alignment tool (Madeira et al., 2019), and alignments were visualized using Jalview 2.11.1.4 (Waterhouse et al., 2009).

Protein structures were predicted using RoseTTAFold (Baek et al., 2021), and the predicted secondary structure was added to the Clustal Omega allele sequence alignment using ESPript 3.0 (Robert & Gouet, 2014). Allele structures were used as a query search against the Protein Data Bank in DALI, and structure pairwise comparison was performed (Holm, 2020). Allele structures were further analyzed and aligned using UCSF Chimera (Pettersen et al., 2004). Allele expression was analyzed by mapping the variable coding regions to the RNA-seq data using Burrows-Wheeler Aligner software package.
(BWA-MEM) (Li & Durbin, 2009), and results were visually examined using the Integrated Genomics Viewer (Robinson et al., 2011).

3.3 Results

Sequencing data reflect phylogenetic relatedness of MRI and SB22

We generated 12.8 million (MRI), 14.3 million (SB22), and 9.9 million (Sporangia) high quality paired-end reads per replicate for three controls (Table 3.1, Supporting Information Figure S1). Considering the increased complexity of infected samples, we doubled the sequence coverage and generated an average 24.8 million and 27.6 million high quality Illumina paired-end reads per replicate per infection sample for MRI and SB22, respectively (Table 3.1, Supporting Information Figure S1). All sequence data were deposited at NCBI under GEO NUMBER: GSE111387.

Table 3.1 Summary of assembled transcripts by timepoint.

<table>
<thead>
<tr>
<th>Dataset (organism)</th>
<th>Read pairs (millions)</th>
<th>Mapping Percentage</th>
<th>Average base quality</th>
<th>Number of Genes</th>
<th>Average gene length</th>
<th>Average coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporangia (P. belbahrii)</td>
<td>29.7</td>
<td>84.1</td>
<td>37.28</td>
<td>10144</td>
<td>1603</td>
<td>230.4</td>
</tr>
<tr>
<td>MRI Water (O. basilicum MRI)</td>
<td>42.7</td>
<td>62.0</td>
<td>37.20</td>
<td>66930</td>
<td>893</td>
<td>66.4</td>
</tr>
<tr>
<td>MRI 12 hpi (O. basilicum MRI, P. belbahrii)</td>
<td>75.9</td>
<td>63.3</td>
<td>37.21</td>
<td>81873</td>
<td>897</td>
<td>98.1</td>
</tr>
<tr>
<td>MRI 24 hpi (O. basilicum MRI, P. belbahrii)</td>
<td>76.3</td>
<td>63.3</td>
<td>37.22</td>
<td>89302</td>
<td>864</td>
<td>92.6</td>
</tr>
<tr>
<td>MRI 48 hpi (O. basilicum MRI, P. belbahrii)</td>
<td>77.0</td>
<td>63.1</td>
<td>37.23</td>
<td>85832</td>
<td>860</td>
<td>98.7</td>
</tr>
<tr>
<td>MRI 72 hpi (O. basilicum MRI, P. belbahrii)</td>
<td>69.8</td>
<td>63.4</td>
<td>37.22</td>
<td>78061</td>
<td>909</td>
<td>93.5</td>
</tr>
<tr>
<td>SB22 Water (O. basilicum SB22)</td>
<td>36.3</td>
<td>62.9</td>
<td>37.18</td>
<td>52844</td>
<td>977</td>
<td>69.9</td>
</tr>
<tr>
<td>SB22 12 hpi (O. basilicum SB22, P. belbahrii)</td>
<td>85.9</td>
<td>63.9</td>
<td>37.18</td>
<td>74918</td>
<td>934</td>
<td>117.6</td>
</tr>
<tr>
<td>SB22 24 hpi (O. basilicum SB22, P. belbahrii)</td>
<td>83.2</td>
<td>63.3</td>
<td>37.19</td>
<td>72899</td>
<td>949</td>
<td>114.5</td>
</tr>
<tr>
<td>SB22 48 hpi (O. basilicum SB22, P. belbahrii)</td>
<td>75.9</td>
<td>65.2</td>
<td>37.15</td>
<td>84478</td>
<td>870</td>
<td>100.9</td>
</tr>
<tr>
<td>SB22 72 hpi (O. basilicum SB22, P. belbahrii)</td>
<td>86.6</td>
<td>66.6</td>
<td>37.20</td>
<td>80675</td>
<td>903</td>
<td>117.7</td>
</tr>
<tr>
<td>MRI Combined Assembly</td>
<td>370.4</td>
<td>64.8</td>
<td>37.22</td>
<td>133,441</td>
<td>765</td>
<td>352.6</td>
</tr>
<tr>
<td>SB22 Combined Assembly</td>
<td>399.6</td>
<td>66.0</td>
<td>37.19</td>
<td>118,296</td>
<td>692</td>
<td>477.7</td>
</tr>
</tbody>
</table>

In total, 240.2 million paired-end reads were used to generate the MRI Combined Assembly, containing 341,633 unique transcripts corresponding to 133,441 genes called by Trinity. The SB22 Combined Assembly was generated using 263.9 million paired-end
reads and contained 118,296 genes and a total of 322,696 unique transcripts. The MRI and SB22 plant-only control assemblies contained 66,930 and 52,844 genes respectively, and the sporangia control contained 10,144 assembled genes. As expected, more genes were assembled in infected samples, representing both host and pathogen transcripts, genes expressed only during infection, and assembly errors (fragmented sequences) introduced as transcriptome complexity increased.

Though fewer reads were sequenced for both water and sporangia control samples, the sporangia control produced the highest sequence coverage (>90x) and longest average gene length (1,603 bp) for assembled genes, likely due to the smaller genome/transcriptome size of the (inactive) pathogen. The average assembled gene length of *O. basilicum* transcripts from a previously published transcriptome was 1,363 bp (Rastogi *et al.* 2014), larger than our average assembly size. The average gene size of the oomycete *Phytophthora infestans* was 1,523 bp (Haas *et al.* 2009), roughly equivalent to the sporangia control assembly.

To assess the genetic diversity between MRI and SB22, we performed a BLAST search between MRI and SB22 water control assemblies and identified 20,943 reciprocal hits, likely representing orthologs between these two plants. These orthologs are highly similar with an average pairwise sequence identity of 98.66% (Figure 3.1B). We further assessed the genetic similarity between the cultivars by conducting a phylogenetic analysis of nine protein-coding chloroplast genome orthologs across members of the asterid clade, to which *O. basilicum* belongs, and with *A. thaliana* and *S. oleracea* set as outgroups (Rastogi *et al.*, 2015). The analysis showed high sequence conservation among the *Ocimum* spp. with MRI and SB22 grouped together (Figure 3.1C). We anticipate that
unique genes or differentially regulated genes are likely to contribute to the MRI and SB22 phenotypic variations.

**Figure 3.1 Basil cultivar phenotypic and genetic diversity.**

A. BDM susceptible ‘Newton’ (SB22) and resistant ‘Mrihani’ (MRI) cultivars. B. Bi-directional blast hit among 20,943 genes shared between the SB22 and MRI water references. The nucleotide identity for each top BLAST hit is graphed here with a bin size of 0.25%. C. Multigene phylogeny of chloroplast genome orthologs across members of the asterid clade.
Using expression profiles in the individual assemblies, MRI transcripts can be divided into 36,414 predicted plant transcripts (present in the MRI water control), 9,988 predicted pathogen transcripts (present in the sporangia control), and 29,502 infection unique transcripts (absent in both plant and pathogen controls) (Table 3.2). Similarly, the SB22 transcripts include 31,702 transcripts with a plant origin, 9,426 transcripts of pathogen origin, and 26,486 transcripts uniquely present in the infection samples. Consistent with SB22 susceptibility, we saw a significant increase in the number of expressed pathogen genes in the susceptible host, observing an almost 20-fold increase from 12 to 72 hpi compared to only a two-fold increase for MRI (Figure 3.2). Measuring total pathogen mRNA abundance (the number of reads mapping to roughly 4,553 pathogen genes), we detected a 43-fold increase in mapped pathogen reads from the SB22 72 hpi samples relative to the MRI 72 hpi samples (Figure S2).
### Table 3.2 Expressed genes across timepoints

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Total Genes</th>
<th>Plant Control</th>
<th>Sporangia Control</th>
<th>Infection Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI Water</td>
<td>36,528</td>
<td>36,414*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRI 12 hours</td>
<td>39,349</td>
<td>29,247</td>
<td>472</td>
<td>9,534</td>
</tr>
<tr>
<td>MRI 24 hours</td>
<td>40,171</td>
<td>29,074</td>
<td>389</td>
<td>10,606</td>
</tr>
<tr>
<td>MRI 48 hours</td>
<td>40,973</td>
<td>28,852</td>
<td>179</td>
<td>11,840</td>
</tr>
<tr>
<td>MRI 72 hours</td>
<td>39,279</td>
<td>28,365</td>
<td>825</td>
<td>9,987</td>
</tr>
<tr>
<td>Sporangia</td>
<td>10,102</td>
<td>0</td>
<td>9,988*</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total unique</strong></td>
<td><strong>76,018</strong></td>
<td><strong>36,414</strong></td>
<td><strong>9,988</strong>*</td>
<td><strong>29,502</strong></td>
</tr>
<tr>
<td>SB22 Water</td>
<td>31,794</td>
<td>31,702*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SB22 12 hours</td>
<td>35,561</td>
<td>25,770</td>
<td>274</td>
<td>9,431</td>
</tr>
<tr>
<td>SB22 24 hours</td>
<td>36,732</td>
<td>25,584</td>
<td>699</td>
<td>10,364</td>
</tr>
<tr>
<td>SB22 48 hours</td>
<td>40,216</td>
<td>25,746</td>
<td>1,788</td>
<td>12,598</td>
</tr>
<tr>
<td>SB22 72 hours</td>
<td>40,838</td>
<td>25,414</td>
<td>5,375</td>
<td>9,962</td>
</tr>
<tr>
<td>Sporangia</td>
<td>9,518</td>
<td>0</td>
<td>9,426*</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total unique</strong></td>
<td><strong>67,706</strong></td>
<td><strong>31,702</strong></td>
<td><strong>9,426</strong>*</td>
<td><strong>26,486</strong></td>
</tr>
</tbody>
</table>

* - When the data was split into 3 sets 114 MRI points and 92 SB22 points were removed due to ambiguity
+ - The numbers displayed in the chart above are total genes in each category as split by FPKM based separation after mapping to each master assembly. If the gene had a non-zero FPKM in the sporangia samples it was included in the "sporangia" sample. The difference in gene count is a reflection of mapping to two separate assemblies.
Figure 3.2. Plant and pathogen gene expression profiles from clustering of the MRI Combined Assembly. Clustering was done using all datasets mapped to the MRI combined assembly. Cluster numbers are displayed above each cluster image. Dataset and replicate are listed on the bottom x-axis. Sporangia expressed gene expression is in red, infection unique gene expression is in gray and gene expression in the mock infected water controls are in black for both MRI and SB22.

*Clustering analysis highlights transcripts with potential functions involved in host-pathogen interactions*

Based on overall ortholog identity, we were confident that SB22 reads from orthologous genes would align to the MRI assembly. Using the MRI Combined Assembly as a reference, we mapped all the SB22 and MRI reads using RSEM with default parameters. Initial coarse clustering using all genes from MRI and SB22 mapped data resulted in 12 clusters with on average 2,810 genes per cluster (Supporting Information Table S2). Three clusters that lacked consistency among biological replicates were not included in further analyses. Three clusters (A1, A2 and A3) characterized transcripts primarily belonging to the pathogen (Figure 3.3 panel A), as all transcripts showed significant expression in sporangia pathogen control samples (Figure 3.3 grey bar
in the middle), but no expression in both water-inoculated plant control samples (Figure 3.3 two blue bars representing MRI-water only and SB22-water only).

Six clusters containing primarily plant transcripts were identified. All transcripts showing significant expression in plant and infected plant samples that were absent from sporangia pathogen control samples were filtered (Figure 3.3 Panels B and C). The remaining genes from the six plant gene clusters represent plant transcripts during pathogen challenge. Transcripts within three clusters, B-1, B-2 and B-3, had comparable expression profiles between MRI and SB22, indicating conserved functions between the two different plant hosts. Both B-1 and B-2 clusters show a pattern consistent with a 12-hour shift in photoperiod, but these clusters respond in opposite directions. Cluster B-1 was upregulated at 24, 48, and 72 hpi and was enriched for metabolism, oxidation-reduction, and photosynthesis functions. No GO terms were significantly enriched in cluster B-2 which showed downregulation at 24, 48, and 72 hpi; however, of those GO terms annotated by Blast2Go metal ion functions were predominant. The largest cluster, B-3, roughly represents stably expressed plant genes. Cluster B-3 is enriched for many categories including various metabolic processes, protein modification, and protein localization, among others.

Transcripts in three clusters, C-1, C-2, and C-3 displayed differential regulation responses between MRI and SB22 during pathogen challenge (Figure 3.3B). In Cluster C-1, MRI expression is high but almost completely absent from the SB22 transcriptome. In Cluster C-2, MRI expression was higher than SB22 and was enriched for genes related to defense response, response to stress, response to stimulus, and DNA integration. In
Cluster C-3, the expression of MRI genes is instead lower than SB22. No GO terms were enriched in cluster C-3.

Figure 3.3 Clusters with expressed plant genes. Nine gene clusters produced by coarse clustering are displayed here. Expression value, y-axis, is on a log2 scale. Three replicates for each dataset are represented by tick marks on the bottom x-axis, with mean data plotted as dark blue points and gray shading representing the standard deviation. Datasets from MRI are highlighted in orange with 3 replicates from 12 to 72 hpi, SB22 datasets are colored pink with 3 replicates from 12 to 72 hpi, both water controls are labeled blue (MRI left, SB22 right), and the pathogen sporangia control data is colored gray. Column A, pathogen gene clusters as all transcripts showed significant expression in sporangia pathogen control samples but were absent from plant and infected plant samples. Columns B and C gene clusters contain primarily plant transcripts as all transcripts showed significant expression in plant and infected plant samples but were absent from sporangia pathogen control samples. Column B, plant genes expressed similarly in both cultivars. Column C, plant genes with different profiles between cultivars.
MRI unique expressed genes include NLR, RLK and secondary metabolic enzymes

To understand potential mechanisms underlying the resistance, we repeated the clustering with higher stringency (p=20, see methods for details) resulting in 188 clusters. Eight clusters were chosen as they were expressed in MRI, minimally expressed in SB22, and showed no expression in the sporangia control. A comprehensive filtering process of the eight clusters resulted in a total 369 MRI unique candidate genes. These MRI unique candidate genes can be grouped into secondary metabolic enzymes (22 genes), immunity related genes including 22 nucleotide-binding site leucine-rich repeat (NLR) genes and 25 receptor-like kinases (RLK) or receptor-like proteins and others.

Detecting secondary metabolic enzymes as MRI unique genes is expected as these two basil plants produce distinct secondary compounds. For instance, SB22 accumulates a significant amount of eugenol, while MRI predominantly accumulates methylchavicol (Rob Pyne, unpublished data). This distinct chemotype prevents MRI from immediate commercial use. Examining twenty-five secondary metabolite related genes predicted as MRI unique genes, we found enzymes related to secondary metabolites which specifically differentiate the MRI and SB22 chemotypes, including cinnamate p-coumarate carboxyl methyltransferase, enzymes involved in anthocyanin biosynthesis, and chavicol/eugenol O-methyltransferase, the enzyme that catalyzes the conversion of chavicol to methylchavicol, as would be predicted from the chemotypes.

Unique expression of RLKs and NLRs, both immunity related protein families, in MRI is of particular interest. Basic plant immunity consists of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006; Cui et al., 2015). Plant PTI uses receptor-like proteins/kinases (RLPs/RLKs), a large gene family that have
roles as sensors of microbe-associated molecular patterns (MAMPs) and induce
downstream defense reactions (Jones & Dangl, 2006; Dodds & Rathjen, 2010; Bigeard et
al., 2015; Chern et al., 2016; Mendy et al., 2017; Zhou & Zhang, 2020). Plant ETI
employs an intracellular nucleotide-binding site and leucine-rich repeat domain receptors
(NLRs) that play roles in sensing effector proteins secreted by pathogens and regulating
downstream defense signaling (Jones & Dangl, 2006; Cui et al., 2015; Cesari, 2018;
Monteiro & Nishimura, 2018; Wang & Chai, 2020).

To investigate basil PTI, we further characterized the RLK BLAST hits. The best
RLK candidate is transcript mri_comp170662, which encodes a full-length malectin-like
RLK. Some predicted receptor-like kinases (RLK) as truncated fragments, such as three
transcripts containing only the leucine-rich repeat (LRR) domain, two containing only
protein kinase domains, and two that were too short to have domain affiliation. Two
transcripts contained an RLK-like domain involved in antifungal and salt tolerance
(Sawano et al., 2007; Zhang et al., 2009). Using the full-length top candidate RLK
transcript mri_comp170662, we identified 12 and 10 homologs in the MRI and SB22
assemblies, respectively. Ten SB22/MRI orthologous pairs can be readily identified
between these two sister cultivars based on a phylogeny (three pair members lacking a
malectin domain were excluded from the tree) (Figure 3.4A). Three MRI orphan
transcripts including this top RLK candidate mri_comp170662 showed increased
expression at 12 and 24 hpi compared to the water control (Figure 3.4B). This RLK
candidate contains LRR, Malectin and kinase domains (Figure 3.4C).
Figure 3.4. Malectin-like RLK proteins in MRI and SB22. A. Alignment of 19 of 22 RLK proteins by their conserved malectin domain. Gray bars with numbers represent orthologous pairs and boxes indicate sequences absent from the SB22 cultivar. B. Fold change compared to water for MRI and SB22 RLKs across infected plant samples. C. Protein domain structure of 22 RLK hits generated from translated nucleotide sequences. Transcript IDs are those of the adjacent fold change row.

NLRs upregulation in MRI during infection

To identify unique MRI NLR resistance genes involved in ETI, we focused on Cluster C-1 where all 17 transcripts are highly expressed in MRI upon infection but almost absent from the SB22 transcriptome. Two transcripts, comp_178221_c0 and comp_160460_c0, are putative NLR resistance genes encoding a late blight resistance protein homolog R1A (gi:848916018 and gi:848932751) from spotted monkey flower (Erythranthe guttata), which belongs to the order Lamiales including basil.

Members of the plant NLR protein family have been characterized as sensors, recognizing specific microbial effectors in response to ongoing host-pathogen...
coevolution, or as helpers involved in signal transduction (Wu et al., 2017). These proteins are highly conserved in eukaryotic immune responses (Wu et al., 2017). NLRs contain a central nucleotide-binding domain and a C-terminal leucine-rich repeat region that confers specificity to the receptor (Wu et al., 2017; Prigozhin & Krasileva, 2020). There are three subfamilies of NLRs defined by presence of one of three functional N-terminal domains: Resistance To Powdery Mildew 8 (RPW8), Coiled-Coil (CC), or Toll/Interleukin-1 Receptor homology (TIR) (Prigozhin & Krasileva, 2020).

The coiled-coil domain NLR subfamily (CC-NLRs) comprises several well-characterized intracellular receptors, including Recognition of Peronospora Parasitica 1 (RPP1) genes conferring resistance to downy mildew in A. thaliana (Krasileva et al., 2010). CC-NLRs are characterized by an N-terminal CC domain, which has been associated with oligomerization in characterized CC-NLRs including barley mildew locus A MLA10 and A. thaliana ZAR1, an NLR that polymerizes to form a plant “resistosome” during the immune response (Maekawa et al., 2011; Adachi et al., 2019).

We focused on validating our computational prediction and further examined our top candidate NLR transcript comp160460_c0 identified in Cluster C-1. Transcript comp_160460_c0, MRI Resistance gene 1 (MRI-R1), encodes a full-length CC-NLR protein of 887 aa with all three functional domains. This NLR candidate is uniquely expressed and differentially upregulated in MRI in the presence of the pathogen (Figure 3.5A). Based on mapping results and expression analyses, while MRI-R1 is expressed in the control samples, its expression is significantly increased in the pathogen-inoculated samples throughout the time-course of infection. Specifically, MRI-R1 was 2-fold upregulated between 12 and 24 hpi and upregulated expression was maintained 48- and
72-hpi. Mapping the SB22 infected assemblies against the MRI-R1 transcript sequence as a reference confirmed that there was no detectable expression of MRI-R1 detected in susceptible samples.

To determine whether MRI-R1 activity could be attributed to presence/absence polymorphism between MRI and SB22 or the differential regulation at transcriptional level, we designed primers to amplify the coding region of the full-length MRI-R1 transcript using PCR. Forward primer 1BF flanks the 5’ end of the coding sequence, and reverse primer 14R targets the 3’ end of the gene. This primer pair produced an amplicon of the MRI-R1 gene from MR1, but not from SB22 or the water template negative control (Figure 3.5B). Internal primers were designed to further examine MRI-R1 like genes in MRI and SB22 (Supporting Information Table S1). PCR using internal forward primer 9F, which flanks the NB-ARC domain, paired with 3’ end primer 14R, resulted in gene amplification from MRI and potentially off-target or ortholog amplification from SB22 (Figure 3.5B). These results suggest that the full MRI-R1 gene is unique to MRI, but that there is some partial sequence conservation in a similar gene of unknown functional status in SB22. Thus, MRI-R1 represents another case of R gene polymorphism among closely related organisms.

The MRI-R1 amplicon detected in the MRI gDNA appeared to be approximately 800bp larger than predicted from the RNA transcripts, indicating the potential presence of a non-coding intronic region (Figure 3.5B). We also observed sequence polymorphisms among MRI-R1 transcripts with 6 different isoforms predicted for the same gene (2 with full coding sequences, comp160460_c0_seq2 and comp160460_c0_seq5). To investigate transcript polymorphisms we isolated and
sequenced individual clones from the PCR products. A total of 12 full-length amplicons were cloned from three MRI plants. Due to the length of the transcript and to help validate the sequences, between 12 to 25 overlapping sequences were generated. Assembly and annotation of these MRI-R1 sequences revealed 4 separate alleles of the gene, supporting the allelic polymorphisms observed in the transcriptomic data (Figure 3.5C). The pairwise nucleotide sequence identity among the four MRI-R1 alleles range from 80% to 96.45%. Alleles 1 and 2 share an identical 827 nucleotide intron sequence while alleles 3 and 4 share an identical 376 nucleotide intron sequences, which accounts for the size discrepancy in the agarose gel electrophoresis result (Figure 3.5B).

Analysis of the protein sequences using InterProScan (Jones et al., 2014) showed that all four alleles contain the CC, NB-ARC, and LRR domains with canonical functional motifs. The N-terminus of all four alleles begins with an identical MADA motif, a functional motif conserved in approximately 20% of CC-NLR immune receptors across distantly related plant species (Bentham et al., 2018; Adachi et al., 2019). The MADA motif has been shown to be necessary for Nicotiana benthamiana NRC4 cell death like the ZAR1 resistosome (Adachi et al., 2019). Similarly, the EDVID motif known to be involved in self-association, direct interactions with cofactors and, in some cases, cell death signaling resulting in a hypersensitive response (Bentham et al., 2018), is also present in all 4 alleles. The NB-ARC domain of all four alleles also contains the Walker A (P-loop) motif (GMFGLGKT) (Ramakrishnan et al., 2002), which is critical for nucleotide binding (Steele et al., 2019). Also present in the four alleles is the MHD-type motif IHD, which has been shown to be involved in inhibition of autoactivation of R proteins in the absence of a pathogen (van Ooijen et al., 2008). The MHD motif is
proposed to act as a molecular switch for R protein activation, and the histidine and aspartate residues are the most highly conserved across R proteins, with the histidine occupying a critical position in an ADP-binding pocket (van Ooijen et al., 2008). The most variable regions among these 4 alleles are in and between the CC and NB-ARC domains (Figure S3).

Figure 3.5 MRI-R1 unique presence, expression, and alleles in MRI. A. Expression of MRI-R1 in *P. belbahrii*-inoculated MRI and SB22 cultivars expressed in TPM (transcripts per million). B. Unique amplification of MRI-R1 from MRI (3673bp) using the external 1BF forward and 14R reverse primers. Internal primer, 9F, paired with 14R produces amplicons of expected size 1206bp in both MRI and SB22. The negative control (NEG) is water in place of gDNA template. C. Gene models of the 4 MRI-R1 alleles with domains and subdomains predicted by InterProScan colored as: coiled coil (gold), NB-ARC (blue), and leucine-rich repeat (green). Introns between the coiled coil and NB-ARC domains are represented as a single line.
Protein structural models of all four alleles were generated in the Robetta server utilizing RoseTTAFold, a top-ranked deep-learning based protein structure prediction method (Baek et al., 2021; Du et al., 2021). The resulting allele protein structures were queried using a distance matrix alignment in the DALI server to search for the closest structural homologs (Holm, 2020).

The resulting top hits for MRI-R1 included plant proteins such as NB-ARC domain from the tomato immune receptor NRC1 (6S2P), LRR receptor-like serine/threonine-protein kinase FLS2 (4MN8), and LRR receptor-like serine/threonine-protein kinase GSO1 (6S6Q). The top structural homologs also included human and animal LRR proteins such as Leucine-rich repeat transmembrane neuronal protein 2 (5Z8X), Dimeric bovine tissue-extracted decorin (1XCD), and human osteomodulin (5YQ5). The top ten structural homologs ranged in Z scores of 18-22.2 (with Z scores above 20 indicating definite homology, and above 8 indicating probable homology), and average deviation in distance between aligned Cα atoms in 3-D superimpositions, indicated by root mean-squared deviation (RMSD) ranged from 2.2-8.5 angstroms (Supporting Information Table S3) (Holm, 2020).

Structural modeling and homology comparison of each allele were used to refine the initial sequence-based boundary predictions of CC, NB-ARC, and LRR domains (Figure 3.6A). All 4 allele structural models were aligned, revealing conservation of predicted functional domain structures and active sites. As expected, based on high sequence identity, all four alleles shared high structural similarity, with slight differences in domain boundaries predicted by the models (Figure 3.6B). Allele expression analysis
showed a clear bias for expression of MRI-R1 Allele 1 by MRI in response to basil
downy mildew infection.

Figure 3.6 MRI-R1 protein structural modeling and alignment. A. Predicted protein
structure of MRI-R1 allele 1 with domains and motifs colored from N to C terminus as
follows: coiled coil domain in yellow, NB-ARC domain in blue, Walker A motif in red,
Walker B motif in purple, and LRR domain in green. B. Alignment of predicted
structures for all 4 MRI-R1 alleles.

The presence of MRI-R1 in two Backcross progenies ‘Devotion’ and ‘Obsession’

To assess the contribution of MRI-R1 in the four new downy mildew resistant
(DMR) basil cultivars that integrated MRI resistance genes, we tested each for the
presence of the MRI-R1 gene. A partial sequence of ObActin, the ubiquitous positive
control, was successfully amplified from both BDM-susceptible SB22, BDM-resistant
MRI, and all four DMR cultivars, while no amplification was detected from the negative
water template control (Figure 3.7). Interestingly, MRI-R1 was detected in only two,
‘Devotion’ and ‘Obsession’, out of the four new DMR cultivars using primers designed
to amplify the full coding region of the gene (Figure 3.7). The two amplicons are estimated to be 3064 bp and 3515 bp, corresponding to allele 4 and allele 1 for ‘Devotion’ and ‘Obsession’, respectively. These amplicon sizes include 24bp of the 5' UTR (beginning with primer 1BF), and the 827bp intron (alleles 1 and 2) or the 376 bp intron (alleles 3 and 4). The six individual clones selected and sequenced from ‘Devotion’ and ‘Obsession’ were uniform, indicating that only one allele of MRI-R1 was passed from MRI to these offspring.

These four new DMR Cultivars were selected from genetic breeding efforts. Briefly, F1 progeny were generated through the MRI (female)×SB22 (male) cross, exhibiting dominant gene action (Pyne et al., 2015). An F2 family was generated after F1 self-pollination and a single resistant individual RUMS469-11 was selected for hybridization with elite sweet basil inbred line ‘SB13’, which demonstrates downy mildew and Fusarium wilt tolerance. Twenty individuals achieving the highest category of reduced disease severity from the RUMS469-11 (female)×SB13 (male) cross were self-pollinated to generate full sibling families evaluated for response to downy mildew. The DMR ‘Devotion’, ‘Obsession’, ‘Passion’ and ‘Thunderstruck’ were selected from these inbred lines. We anticipate these four new selected DMR cultivars should inherit genetic resistance genes from both MRI and SB13.
Figure 3.7 MRI-R1 detection in DMR offspring. Gel electrophoresis showing amplification of ObActin fragment positive control from all basil cultivars tested on the left, MRI-R1 amplification from ‘Mrihani’ (MRI), ‘Devotion’, and ‘Obsession’ on the right using the external 1BF forward and 14R reverse primers. The negative control (‘control’) is water in place of gDNA template.

Differential upregulation of salicylic acid biosynthesis pathways in MRI

Both NLR and RLK genes interact with hormone signaling pathways to activate host defense (McHale et al., 2006). To understand the involvement of plant hormone signaling pathways involved in susceptibility and resistance responses, we examined the expression of genes involved in ethylene, jasmonic acid (JA), abscisic acid (ABA), jndole-3-acetic acid (Auxin), gibberellic acid (GA), and salicylic acid (SA) based on A. thaliana annotation (1e-20, sequence similarity >40%). No significant differences were observed between the MRI and SB22 pattern of expression in ethylene, jasmonic acid, abscisic acid, or gibberellic acid pathway genes (Figure S4, Table S2). We saw a difference in expression profile for two of five auxin genes; YUC1 and TAA1 both were upregulated at early timepoints in MRI, however they were not statistically significantly differentially expressed (p value > 0.05).
Figure 3.8. MRI and SB22 diverge transcriptionally at salicylic acid synthesis. Bar graphs are the average and standard deviation of three replicates. White bars indicate MRI data and gray bars indicate SB22 for each timepoint. Infection releases the repression WRKY70 has on SARD1 expression. Double arrows leading from SID2 and PAL indicate more than one step to the SA molecule.

The most striking difference was observed among genes required for the synthesis of salicylic acid (Figure 3.8). Plants possess two biosynthesis pathways to synthesize SA, both starting from chorismate, but subsequent steps involve either isochorismate synthase (ICS) or SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2) (Wildermuth et al., 2001) and phenylalanine ammonia-lyase (PAL) (Olsen et al., 2008). There is no significant difference in PAL expression between the two cultivars, but we did observe a
drastic induction of ICS 24 hpi in MRI. For the water control and 12 hpi samples, there was no significant difference in ICS expression between MRI and SB22. Compared to SB22, ICS expression in the MRI cultivar is 15, 6 and 5-fold higher at 24, 48 and 72 hpi, respectively.

SARD1 is upstream of SID2 in the SA synthesis pathway, and the average SARD1 expression across all time points is 7-fold higher in MRI compared to SB22 and has roughly 2-, 2.5-, 0- and 3.6-fold increases at 12, 24, 48 and 72 hpi compared to the water control. Similarly, PR1, a commonly used marker gene downstream of SA synthesis, is significantly induced in MRI upon pathogen challenge starting at 24 hpi, confirming the unique increase of SA in MRI upon pathogen challenge.

Together, our data suggest that MRI-derived BDM resistance involves SA. The most significant change occurred 24 hpi, one day after the encounter of the plant with the pathogen. WRKY70 is known to repress SARD1 expression in the absence of pathogens and is required for the activation of some defense genes (Li et al., 2004; Zhou et al., 2018). In MRI, we identified a strong induction in the transcription of WRKY70 at 12 hpi, increasing expression of SARD1 from water to 24 hpi, and a 23-fold rise in the relative normalized expression of SID2 at 24 hpi relative to 12 hpi. In SB22, we observed only a slight increase in WRKY70 expression which was delayed until 24 hpi, and SID2 expression rose only 2.5-fold by 24 hpi and remained effectively stable at later time points. Similarly, we saw a roughly 2-fold induction in the expression of PR1 in MRI between 12 hpi and 24 hpi, consistent with the upregulation of SID2, while at the same time there was no increase in PR1 expression in SB22 between 12 and 72 hpi.
3.4 Discussion

Here we report the results of transcriptomic sequencing of basil cultivars infected with *Peronospora belbahrii* with the goals of identifying genes conferring resistance and understanding what pathways are involved in susceptibility. We sequenced 33 datasets to a reasonable coverage, likely capturing most transcripts, although total gene length was shorter than a previous study in sweet basil (Rastogi *et al.*, 2014), and Trinity’s estimate of gene count in the plant samples was greater than expected. Nonetheless, we identified roughly 21,000 orthologous genes between cultivars with overall high sequence conservation consistent with closely related individuals. Phylogenetic analysis supports the hypothesis that SB22 and MRI are closely related, interspecific genotypes, which was previously shown using SSRs to confirm conservation of the orthologs in the two breeding parents (Pyne *et al.*, 2018). These results underscore the close relationship between cultivars and their sexual compatibility, likely providing us with a pool of true genes shared in MRI and SB22. At the time of this study, no sweet basil genome was available for use in a reference-based assembly, though a draft genome for Genovese-type cultivar ‘Perrie’ has since been published (Gonda *et al.*, 2020). Furthermore, several unique MRI gene candidate sequences were used to search the *O. basilicum* draft genome and no significant BLAST hits were retrieved.

Gene expression clustering identified patterns consistent with MRI unique infection-expressed genes. Annotated MRI unique candidates were found to be enriched for NLR, RLK, and secondary metabolic proteins. Analysis of the NLR and maelectin-like RLK genes in both MRI and SB22 identified orthologous pairs and cultivar-unique genes. Members of the NLR and RLK families are known to act alongside or upstream of
disease signaling pathways and serve as good candidate resistance genes. Secondary metabolite genes identified in the two cultivars correlate well with known chemotypic characteristics differentiating MRI and SB22. The top candidate NLR prediction is supported by PCR screening revealing its presence in resistant MRI and absence in susceptible SB22. This result was further strengthened with the cloning and sequencing-based confirmation of MRI-R1 alleles, as well as predicted amino acid sequence analysis and protein structure modeling supporting the hypothesis that this is a unique NLR likely involved in immune responses to pathogen infection.

The presence of MRI-R1 was detected in only two out of the four resistant cultivars, ‘Devotion’ and ‘Obsession’. However, recent quantitative trait loci (QTL) analysis detected at least two major genomic regions (LOD>4.0) that control DM resistance in the MRI x SB22 F2 mapping population (Pyne et al., 2017), suggesting that the predicted involvement of MRI-R1 in quantitative disease resistance may be redundant or have shared function(s) with other NLRs and/or RLKs. We cannot conclusively determine the functionality of the alleles simply based on the bias for MRI-R1 allele expression in MRI. The presence of Allele 1 in ‘Obsession’ and Allele 4 in ‘Devotion’ indicates that there may be functional redundancy, and further understanding of the conserved motifs in these alleles suggests that there may be interacting partners that have an impact on the activity of these proteins. Nevertheless, we are confident that our transcriptomic pipeline is powerful in detecting resistant genes involved in the host-pathogen interactions.

In addition to prediction of specific genes likely conferring resistance, this comparative transcriptomic approach was also valuable in revealing physiological
mechanisms involved in basil downy mildew resistance. Salicylic acid signaling is an integral part of plant defense responses and has been demonstrated to be involved in defense against downy mildews and other biotrophic pathogens (Delaney Terrence P. et al., 1994; Mohr et al., 2010). Pathogen have developed virulence strategies to overcome and inhibit salicylic acid defenses, thus enhancing susceptibility to biotrophic pathogens such as downy mildew organisms (Caillaud et al., 2013, 2016). This suggests a likely role of salicylic acid signaling in MRI BDM resistance, though the specific mechanisms of signaling induction remain unknown. We hypothesize that multiple NLRs and RLKs are active and have interacting and/or redundant roles in mediating the SA signaling pathway in MRI. If this hypothesis is correct, utilizing multiple targets in a marker-assisted selective breeding program will be more effective and robust to pass resistance from parents to progeny in order to slow the evolution of new pathogen races and/or pathotypes.

This study has utilized mRNA sequencing over an infection time course to provide strong evidence linking susceptibility to known mechanisms which control defense responses, and prediction of genes regulating the resistant phenotype. Transcriptomics without the need for a reference genome is a powerful tool for comparative analyses given the availability of methods for data annotation and pattern identification. The strong resistance phenotype of MRI compared to SB22 likely led to the strength of the visible signal between the cultivars. NLRs that confer resistance to biotrophic pathogens have increased genetic diversity, and here we observe that, despite high genetic conservation between orthologs (Figure 3.1), there are distinct genetic differences in the NLR repertoire (Van de Weyer et al., 2019).
Breeding cultivars with quantitative resistance has been shown to produce durable resistance, typically through the activity of multiple minor-effect genes (Brown, 2015; Niks et al., 2015). Other BDM resistant cultivars have been produced through interspecific hybridization of *O. basilicum* with *O. americanum* var. *pilosum*, leading to dominant resistance against two races of *P. belbahrii* (Ben-Naim & Weitman, 2021). We have observed that the downy mildew-resistant basil cultivars succumb to infection in production systems in different regions, consistent with the report of emerging pathotypes and/or races (Ben-Naim & Weitman, 2021). Therefore, understanding the physiological and molecular bases of host-pathogen interactions is critical to rapidly developing improved cultivars, monitoring strategies, and management practices. Identification of suitable molecular markers conferring multiple sources of resistance will improve breeding for basil downy mildew resistance and advance the understanding of molecular mechanisms of resistance and pathogenicity. These developments in approaches and knowledge can be broadly utilized by plant breeders and pathologists working with downy mildew pathogens on many different crops. After further validation, the genes predicted here can likely serve as molecular markers for the selection of downy mildew resistant sweet basalms, and investigation and validation of physiological responses to infection may aid in developing more robust phenotyping assays. This comparative transcriptomics approach not only revealed candidate resistance genes, but also offered us new insights into differential infection responses in resistant and susceptible cultivars, which will open new avenues of investigation to further combat basil downy mildew.

This method of analysis is broadly applicable to two-organism biological systems where the identification of genes involved in any specific interaction is desired. Although
genomes for holy basil (*O. tenuiflorum*) had been published prior to this study and sweet basil (*O. basilicum*) and *P. belbahrii* genomes have been published since, the methods described here worked exclusively from RNA sequencing data and did not require the whole genome sequence. Any system utilizing two organisms from different kingdoms could be examined using these methods as DNA sequences are differentiable down to reasonable taxonomic levels.

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**Author Contribution**

JS, L-JM, RP, and LG participated in the designing of the experiment. RP prepared all materials for the RNAseq, GAD performed all RNA-seq data analysis. KSA performed phylogenetic analysis and R-gene cloning, sequencing, and protein structural
analyses. AG directed protein structural and sequence analysis. JM provided initial protein structural modeling analysis. KSA, GAD and L-JM wrote the manuscript. KSA and GAD prepared the figures, and all authors edited the paper.

Disclaimer: This article was prepared while Anne Gershenson was employed at the University of Massachusetts Amherst. The opinions expressed in this article are the author's own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.
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McGrath MT. 2021. Where in the USA is Basil Downy Mildew?


Supporting Information

**Table S1** Primers Used In This Study

**Table S2** Coarse Clustering Results

**Table S3** Top ten structural homologs of MRI-R1 identified by DALI PDB Search

Supporting Tables can be found in the preprint:
https://www.biorxiv.org/content/10.1101/2022.05.23.491563v1.supplementary-material?versioned=true.

**Figure S1** Average Quality Scores. Average quality scores plotted on the Y-axis by base number on the X-axis for all paired-end reads.
**Figure S2 Pathogen Read Percentage.** Percentage of reads mapped to the *Peronospora belbahrii* sporangia data in MRI (black bars) and SB22 (grey bars) combined data at each timepoint plotted on the X-axis.
CHAPTER 4

CONCLUSIONS

4.1 LONGEVITY OF DETACHED *P. BELBAHRII* SPORANGIA IS INFLUENCED BY TEMPERATURE AND HUMIDITY

Basil downy mildew remains the most significant disease challenging sweet basil (*Ocimum basilicum*) production worldwide. The causal organism *Peronospora belbahrii* continues to spread to new locations and remains active or is reintroduced in locations where it was previously reported. Infective sporangia of the pathogen are aerially-dispersed and may remain viable and infective for extended periods of time. Our studies addressed the proper handling of the pathogen to produce consistent inoculations in greenhouse settings and identify long-term survival of *P. belbahrii* sporangia.

We established a reproducible inoculation protocol and refined *P. belbahrii* sporangia collection procedures, resulting in an effective study system. Utilizing an *in vitro* sporangia germination protocol and *in planta* inoculations, we determined that *P. belbahrii* sporangia, detached and isolated from the host, remained viable and infective for up to 264 hours at a high relative humidity (96.5% RH) and moderate temperature (20°C). Germination and infectivity of sporangia was reduced at higher temperatures (≥25°C) and lower humidity (84% RH). This result is in agreement with previous studies that have shown that temperature and humidity impact sporangia survival. Our findings represent a likely maximal survival period of detached sporangia, given that we did not expose the sporangia to UV light radiation or fluctuating environmental conditions that would more realistically mimic natural settings. These results may overestimate this period with regards to other environmental factors that reduce survival, but nonetheless
are informative and practical for basil growers to consider when disinfesting growing spaces. Combining destruction of affected crops with reduced humidity and increased temperatures will provide a less favorable environment for basil downy mildew infection. Under typical greenhouse conditions with relative humidity below 84%, greenhouse growers who have removed and destroyed infected plant material should be able to establish new crops as early as 72 hours after sporulation.

4.2 NEW PATHOTYPES OF P. BELBAHRII IN THE US WITH DIFFERENTIAL VIRULENCE PATTERNS ON DISEASE-RESISTANT BASIL CULTIVARS

Downy mildew-resistant basil cultivars have only recently been developed resulting in two main sources of disease resistance which have been introduced in global basil production since 2018. Several cultivars with qualitative, major-gene mediated resistance have been developed through the introgression of non-host resistance genes from Ocimum americanum and subsequent crosses. Additionally, four cultivars were developed with quantitative disease resistance derived from ornamental selection ‘Mrihani’. Accompanying the introduction of resistant cultivars in basil production is the inevitable development of new pathotypes with the ability to “break” or overcome resistance.

Collecting P. belbahrii isolates from a variety of basil cultivars revealed the activity and predominance of resistance-breaking pathotypes in multiple locations and years. We sought to improve the understanding of pathotype virulence patterns by utilizing greenhouse studies to establish differential panels of basil cultivars. These cultivar trials identified three distinct virulence pathotypes, one with the ability to overcome qualitative non-host resistance, and another with increased virulence on
cultivars with quantitative resistance. Interestingly, the pathotype that overcame O. americanum-derived non-host resistance was somewhat controlled in the quantitative resistant ornamental cultivar ‘Mrihani’, as well as one of the progeny with quantitative resistance. This clear divergence of virulence suggests that the *P. belbahrii* pathotypes have developed multiple strategies for resistance-breaking. Further analysis of the underlying genetics will be explored to identify these strategies. These results also demonstrate the need for multiple sources of resistance to be combined for improved disease-resistant basil breeding.

### 4.3 DISEASE-RESISTANT BASIL ‘MRIHANI’ DEMONSTRATES QUANTITATIVE RESISTANCE DURING *P. BELBAHRII* INFECTION, POTENTIALLY INVOLVING CANDIDATE RESISTANCE GENE MRI-R1 AND SALICYLIC ACID BIOSYNTHESIS AND SIGNALLING

Basil breeding and quantitative trait locus analysis previously confirmed the genetic basis for quantitative disease resistance in ‘Mrihani’, which was crossed with susceptible ‘Newton’ in a multi-generational breeding program to produce new cultivars with improved disease resistance and other horticultural traits. The causative genes at the quantitative loci were unidentifiable due to the lack of genetic resources for basil. To overcome this knowledge gap, a comparative transcriptomic analysis was designed to identify genes and elucidate mechanisms controlling quantitative disease resistance.

The comparative transcriptomic responses of ‘Mrihani’ and ‘Newton’ revealed many global differences in disease resistance and susceptibility responses. Candidate resistance genes were identified by filtering the data for transcripts with increased expression in ‘Mrihani’ during infection, and with predicted domains, motifs, and/or
homology to known genes involved in plant defense responses. Among ninety-five candidate resistance-related transcripts, candidate gene MRI-R1 was chosen as the top candidate nucleotide-binding leucine-rich repeat (NB-LRR)-type resistance gene. ‘Mrihani’. Resistant progeny were screened for the presence of MRI-R1, and it was found to be present in ‘Mrihani’, with four alleles in the allotetraploid parent background, and single alleles found in two of the resistant progeny. Protein structural modeling and sequence analysis bolstered the hypothesis that MRI-R1 encodes a canonical NB-LRR disease resistance protein.

Examination of classical defense responses identified the unique hormone signaling profile of salicylic acid in ‘Mrihani’. These results suggest a likely role of multiple disease resistance genes with interacting partners and/or overlapping functions, including MRI-R1 in mediating quantitative disease resistance, with downstream salicylic acid signaling as a potential mechanism. This pathosystem is complex, and it is unrealistic to perform conclusive studies of individual genes or isolated mechanisms at this time, but our studies have unraveled potential genes and mechanisms of quantitative disease resistance by utilizing comparative transcriptomics to amplify the signal(s) of plant responses during pathogen challenge.

Taken together these studies address the agricultural challenge of controlling basil downy mildew by examining pathogen epidemiology, virulence, and host resistance. This research has established the basis for multiple interesting and timely continued investigations, including the refined identification of breeding markers, comparative genomics of pathogen populations, and identification of virulence mechanisms.
APPENDIX A. RUTGERS BASIL BREEDING SCHEME
APPENDIX B. BASIL DOWNY MILDEW INOCULATION PROTOCOL

Protocol

1. Place infected plants in a humidity chamber 12 hours prior to inoculation (overnight) to force sporulation.

2. Preparation of fresh *P. belbahrii* sporangia:

   a. Prepare 2-3 50 mL conical tubes per sporulating plant, each containing ~35 mL sterile deionized water (dH$_2$O)
      i. If collecting from a large number of leaves, prepare a sterile glass bottle with 200mL of sterile dH$_2$O
   b. Pick off sporulating leaves and place into conical tubes (or sterile bottle)
   c. Shake leaves in conical tubes vigorously to release the sporangia
   d. Place a funnel inside an Erlenmeyer flask and line the funnel with a double layer of autoclaved cheesecloth, then pour all leaf suspensions into the funnel
   e. Re-filter suspension through a quadruple layer of autoclaved cheesecloth to remove additional soil particulates, sporangiophores, etc.
   f. Pour double-filtered sporangia suspensions into 50mL conical tubes and adjust with sterile dH$_2$O to balance
   g. Centrifuge prepared sporangia suspensions for 2 minutes at 4000rpm to pellet sporangia
   h. Pour off supernatant from pelleted sporangia and discard
i. Resuspend sporangia pellets in sterile diH₂O and repeat step 2g centrifugation to wash sporangia (the supernatant from this wash step should be much cleaner in appearance)

j. Resuspend sporangia pellets in ~5mL sterile diH₂O and combine all washed and filtered suspensions into a single 50mL conical tube, swirl vigorously and mix by pipetting

k. Extract 100µL of well-mixed sporangia suspension and load into hemocytometer for quantification

  i. If the sporangia suspension is very dark, it is highly concentrated.
     An aliquot should be taken out and diluted to 1:10 or 1:100 so that sporangia can be accurately counted

l. Assess concentration using hemocytometer and adjust the sporangia suspension to 1x10⁴ sporangia/mL*

m. *If sporangia suspensions are also being prepared for DNA extraction, adjust concentration to 1x10⁶ sporangia/mL and removed the desired number of aliquots, then dilute the remaining suspension for inoculation

n. Prepare spray bottles with inoculum (generally use 5-10 mL suspension per plant)

3. Spray plants with fresh inoculum until run-off (~5mL per plant)

4. Place plants in humidity chamber for 24 hours, then remove and place on a greenhouse bench or in a growth chamber, evenly spaced to reduce the chance of sporulation
5. Plants should begin to show chlorosis at the sites of infection after 4-5 days, at which time sporulation can be forced
   a. For maintenance of the pathogen, plants can remain infected for 2-4 weeks before dropping chlorotic leaves, and sporulation should be forced and new plants inoculated while there are still ample chlorotic leaves
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