# AN ABSTRACT OF THE THESIS OF

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Title: Development of Diagnostic Assays for Race Differentiation of *Podosphaera macularis*.

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David H. Gent

Hop powdery mildew (*Podosphaera macularis*) was confirmed in the Pacific Northwest in 1996. Before 2012, the most common race of *P. macularis* was able to infect plants that possessed powdery mildew resistance based on the R-genes Rb, R3, and R5. Post 2012, two additional races of *P. macularis* were discovered that can overcome the resistance gene R6 and the partial resistance found in cv. 'Cascade'. These three races now occur throughout the region, which can complicate management and research efforts because of uncertainty on which race or races may be present on susceptible cultivars and other germplasm. Current methods for race determination for *P. macularis* are slow, costly, and labor intensive. We sought to develop a molecular assay to differentiate races of the fungus possessing virulence on plants with R6, dubbed V6-virulent, from other races. The transcriptomes of 46 isolates of *P. macularis* were sequenced to identify loci and variants unique to V6-isolates. Fourteen primer pairs were designed for 10 candidate loci that contained

single nucleotide polymorphisms (SNP) and short indels. Two differentially labeled locked nucleic acid probes were designed for a contig that contained a conserved SNP associated with V6-virulence. The resulting multiplexed real-time PCR assay was validated against 46 V6 and 54 non-V6 *P. macularis* isolates collected from the United States and Europe. The assay had perfect discrimination of V6-virulence among isolates of *P. macularis* originating from the western U.S. but failed to predict V6-virulence in three isolates collected from Europe. The specificity of the assay was tested with other powdery mildew species and pathogens of hop. Weak non-specific amplification occurred with powdery mildew collected from grape, strawberry, and zinnia; however, non-specification amplification is not a concern when differentiating pathogen race from mildew colonies on hop. The assay has practical applications in hop breeding, epidemiological studies, and other settings where rapid confirmation of pathogen race is needed.

# Development of Diagnostic Assays for Race Differentiation of *Podosphaera macularis*

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Mary H. Block, Author

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# CONTRIBUTION OF AUTHORS

David Gent was involved with all aspects of these studies. Brian Knaus and Nik Grünwald conducted the assembly and analysis of the Illumina data used for assay development. Michele Wiseman assisted in trouble shooting molecular assays and training in hop and powdery mildew maintenance.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF TABLES

# Chapter 1: Introduction and Literature Review

## Rationale for Research

The Pacific Northwest of the United States is known for its craft brewing industry with 284 craft breweries located in Oregon alone (Brewers Association 2020). The states of Oregon, Washington, and Idaho fall within the optimum growing range for hop and produce over 98% of the hops harvested in the United States (USDA NASS 2019). Hop plants are clonally propagated, so consequently, the cultivars used are generally uniform and there is homogeny within fields. Further, the demands of the brewing industry dictate which cultivars are planted (Haunold 1981). This combination leads to an increased risk of disease susceptibility due to the preference for brewing attributes and quality over disease resistance.

An important disease risk is hop powdery mildew, caused by *Podosphaera macularis* Braun & Takamatsu (formerly *Sphaerotheca macularis* (Wallr. :Fr.) Lind, syn. *S. humuli* (DC.) Burrill), which is one of the most destructive diseases of hop and can destroy an entire crop if left uncontrolled. Powdery mildew has been present on hop in the Pacific Northwest since the late 1990's (Ocamb et al. 1999). New races of powdery mildew have arisen in response to the release and wide distribution of new hop cultivars (Gent et al. 2017; Wolfenbarger et al. 2016). Current methods for race detection in the hop powdery mildew fungus are labor intensive, time consuming, and costly. A rapid detection method for races of the fungus would benefit producers, researchers, diagnosticians, and plant clinics, and could aid in disease management

decisions for growers. Herein, this thesis describes development and validation of such an assay.

**Hop, *Humulus lupulus* L.**

The cultivated hop, *Humulus lupulus* L., has been grown for centuries for the preservative and bittering qualities of the female inflorescence informally known as the hop cone (Wilson 1975; Zanoli and Zavatti 2008). *Humulus lupulus* belongs to the family Cannabaceae, along with *H. scandens* and *H. yunnanensis*. These three *Humulus* species are believed to have originated in China with later introductions of *H. lupulus* to Europe, Japan, and North America (Neve 1991; Murakami et al. 2006). *Humulus lupulus* is a dioecious, perennial vining plant with annual bines that climb by wrapping around and clinging to a suitable support in a clockwise direction (Neve 1991). Hop production occurs at latitudes between 35-55 degrees north and south due to day length requirements for optimal and synchronous flowering. Plants are cultivated on approximately 6-meter tall trellises and enter into a period of dormancy in the fall following harvest (Beatson 2005). Dormant periods vary in length between England, continental Europe, and the United States, and also among cultivars. Hop plants can grow up to 2 meters a week during the peak growing season. It can take up to three years for the plant to reach maturity depending on environment (Burgess 1964), although in the Washington State growing regions hop plants can reach maturity in as little as two years. Due to the cost of infrastructure required to harvest and maintain hop, brewer quality demands, and the limited range for optimal

production, it is crucial to control diseases that affect hop cone yield and quality (Neve 1991).

Hop plants are normally diploid with a base number of 10 chromosomes although ploidy variations can occur (Haunold 1968). *Humulus lupulus* have both autosomes and sex chromosomes. Only female hop plants are cultivated for the economically important cones. The presence of males near a hop yard can lead to unintentional pollination and seed formation, which lowers the brewing value of the cones (Jakse et al. 2008). Triploid hop cultivars have been produced with the intent of preventing the formation of seed and as a means to quickly reproduce favorable brewing characteristics found in the mother (Haunold 1971; Koutoulis et al. 2005). Despite this effort, globally the majority of hop cultivars are diploid.

Hop replaced the traditional herbs used in the brewing of malt-based beverages around the 13th century in Europe (Moir 2000). The lupulin glands of hop cones contain aromatic compounds that impart bitterness along with unique aroma and flavors to beer. Additionally, hop cones have preservative characteristics and contribute to foam development and stability (Bamforth 1985; Smith et al. 1998). Hop cone quality is based in part on color and aroma, and is subject to the buyer and market demands. Total oil content, alpha-acid levels, and dry matter are standard characteristics used to determine the value of cone lots. The alpha-acid content of hop cones and the subsequent isomerization of the compounds during the wort boiling stage of brewing contributes to the bitterness of beer. Alpha-acids are a major component of hop quality that buyers use when selecting hops because brewers maintain consistency in recipes by using specific percentages of alpha-acids. The oil

component contains terpenes, the aromatic components of hop cones. Exploration of hop aroma compounds have elucidated the characteristic notes that aldehydes, esters, and terpenes contribute to beer flavor. For example, the compounds, myrcene and linalool, are found to impart resinous and citrus flavors to beer (Schönberger and Kostelecky 2011; Stevens 1967). Researchers have also been exploring the properties of these secondary metabolites for use in medical therapies intended for cancer treatment (Nuutinen 2018; Stevens 1967; Zanoli and Zavatti 2008).

Commercial hop cultivars are derived from European and wild American germplasm. European cultivars are known for their aroma characteristics whereas North American hops are distinguished by the high levels of alpha-acids in the lupulin glands. There is a long history of hop cultivation in Europe (Stajner et al. 2008). Hop plants were first introduced into the eastern United States in the mid-17$^{th}$ century with the arrival of British and Dutch colonists (Burgess 1964). By the early 1900's, the American hop industry was well established in New York State.
The hop industry relocated to the western United States in an attempt to survive prohibition era restrictions and to escape the newly-introduced pathogen, *P. macularis* (Barth et al. 1994; Neve 1991).

The Pacific Northwest hop growing region encompasses the states of Washington, Oregon, and Idaho. The Yakima Valley of Washington is the largest hop producing region in the nation and is characterized by a high desert climate. This region is contrasted with the temperate maritime climate of the Willamette Valley of Oregon. The hop growing region of Idaho is primarily localized west of Boise and is characterized by continental climate. While these regions experience different levels

of disease due to disparities in control methods and climate, hop powdery mildew remains one of the most destructive pathogens of hop throughout the Pacific Northwest (Mahaffee et al. 2003a).

**Powdery Mildew Fungi**

Powdery mildew infections are caused by fungi that belong to the subphylum, Ascomytina, under the order Erysiphales (Braun et al. 2002; Glawe 2008). These fungi are characterized by generally white, filamentous colonies found on the leaves and stems of host plants. The name powdery mildew refers to the appearance of the asexual spores. These fungi are obligate, biotrophic plant pathogens, meaning they require living host tissue in order to reproduce. Although these fungi are known to infect thousands of species of angiosperms, each fungal species typically has a very narrow host range. However, there are exceptions to this such as the fungus that causes powdery mildew of onion, *Leveillula taurica*, which can infect other *Allium* species, cucurbits, and solanaceous species (Correll 1987). During favorable conditions, powdery mildew fungi can go through 20 generations in a growing season with a single conidium generating approximately $3.53 \times 10^5$ conidia/$cm^2$ of colony area (Blodgett 1913). The potential for large quantities of inoculum illustrates the necessity of disease control for powdery mildews. Powdery mildew infections can have a large impact on crop production by reducing yield and quality of the product if no disease control interventions are enacted on susceptible plants. In field crops, such as barley, powdery mildew infections affect yield through reduced photosynthetic activity (Gaunt 1995). Powdery mildews can reduce quality by directly infecting the

economically valuable parts of the plant such as the fruit of grapevines or flowers of ornamentals. In horticultural crops, such as pear and apple, the damage of powdery mildew is less apparent if the fruits are not directly infected but the overall health of the tree may be impacted (Jarvis et al. 2002).

It is important to understand how various species of powdery mildew fungi are related in order to correctly identify a potential disease threat and act accordingly by implementing control measures. There are currently16 accepted genera within the Erysiphales, with at least 900 species of powdery mildews recognized (Takamatsu 2013). The historical taxonomic classification of powdery mildew fungi was based on morphological characteristics of sexual ascocarps, namely the structure of chasmothecia appendages and number of asci per fruiting body. Advancements in scanning electron microscopy and molecular technology has allowed for more precise classification of powdery mildews (Braun et al. 2002). The genus, *Podosphaera*, was introduced worldwide by Kunze in 1823 and is distinguished by having external mycelium, conidia with fibrosin bodies and formed in chains, and chasmothecia with dichotomously branched appendages (Neve 1991; Glawe 2006). Modern examinations of the anamorphic and teleomorphic characters of powdery mildew fungi and phylogenetic analysis has resulted in the reduction of *Sphaerotheca* and *Podosphaera* into a single lineage (Braun and Takamatsu 2000). In the Pacific Northwest, powdery mildew species belonging to the *Podosphaera* genus infect important specialty crops such as apple (*P. leucotrica*), cherry (*P. clandestina*), strawberry (*P. aphanis*), and hop (*P. macularis*).

On hop, powdery mildew is initially characterized by small blisters on the leaves due to hypertrophy of the cells around the infection site (Neve 1991). During the early stages of infection, the powdery appearance of the colony is caused by chains of conidia that have grown from the mature mycelium. Diseased cones can appear white from the conidia or develop a rusty red color when ascocarps of the fungus are present. Decreases in photosynthetic activity from relatively small amounts of the disease are believed to have little impact on the hop plant due to the extensive size of the hop canopy. However, infection of the female inflorescence can have devastating effects on crop production by damaging the hop cones to the extent that they are no longer marketable (Gent et al. 2007, 2014, and 2018).

Powdery mildew was first documented on hop in Europe by Worlidge in 1669 (Neve 1991). Powdery mildew was first reported in New York State in 1909 (Blodgett 1915). In 1996, hop powdery mildew was first observed in commercial hop fields in the Pacific Northwest, and became epidemic in Washington State in 1997 (Ocamb et al. 1999). Powdery mildew was documented in the Willamette Valley of Oregon and in the hop growing regions of Idaho in 1998. By 1999, the majority of hop acreage in Washington was affected by the disease (Ocamb et al. 1999). Growers were unprepared for the powdery mildew outbreak and experienced severe losses as a result. In 1999 and 2000, economic losses due to hop powdery mildew and its control were estimated to be 15% of the total crop revenue in the PNW hop growing region (Turechek et al. 2001).

**Powdery Mildew Damage on Hop**

Annual losses attributed to damage caused by hop powdery mildew and costs for its control are estimated to be 15% (Mahaffee et al. 2003a). Hop powdery mildew infections contribute to economic loss due to declines in cone yield and quality. Early infection of developing inflorescences results in the most severe reduction in cone yield. Occurrence of hop powdery mildew at bloom through the early stages of cone development can result in malformed cones or abortion of the flower. Control of hop powdery mildew at Stage I and II of hop cone development (Kavalier et al. 2011) minimizes the impact of the disease on yield (Gent et al. 2014; Twomey et al. 2015). Later infections of cones by the powdery mildew fungus can lead to reductions in yield due to an increased rate of cone senescence. Heavy powdery mildew occurrence also indirectly causes yield losses by increasing dry matter content of cones, which results in increased shattering of cones during mechanical harvesting (Gent et al. 2014).

In addition to yield loss, powdery mildew can reduce the quality of hop cones. Severe powdery mildew infections can cause malformed and discolored cones while moderate infections can cause browning of the cones during the drying process. Cones that have been damaged by powdery mildew may be rejected by brewers due to their discoloration or perceived off-aromas (Neve 1991; Mahaffee et al. 2003a). There is concern that diseased marketable products such as fruits and flowers can lead to quality defects after processing. In both wine grapes and hops, powdery mildew may impact the quality of the finished product. In wine grapes, heavy powdery mildew leads to a viscous mouthfeel and musty flavors in wine (Stummer et al. 2005). There has been debate over whether powdery mildew affects the quality of beer. A

sensory panel was unable to discern any defects in beer in a study performed by Hysert et al. (1998) where cones lightly and heavily infected with powdery mildew were used early in the brewing process. Although it is not apparent if powdery mildew infections on hop cones are perceptible in beer, it is noteworthy that increases in disease can lead to a decrease in alpha-acids and hop oil content (Gent et al. 2014).

**Powdery Mildew Reproduction**

Understanding the reproductive cycle of powdery mildew fungi allows for a more informed response to disease management. Sexually-reproducing fungal pathogens are thought to have the potential to overcome host resistance in plants at a faster rate than asexually reproducing pathogens due to recombination which results in new gene combinations (McDonald and Linde 2002). Powdery mildew fungi have a polycyclic disease cycle and have both anamorphic (asexual) and teleomorphic (sexual) stages. While the sexual cycle has the potential to generate new genetic combinations that can lead to increased virulence or fungicide resistance, the asexual cycle is responsible for the large production of inoculum and dissemination of the pathogen.

In powdery mildew fungi, the asexual cycle is initiated by the elongation of vegetative hyphae into specialized structures called conidiophores which can form several days after infection. Conidia are uninucleate, single cells with large vacuoles that form successively at the base of each propagule (Glawe 2008). There is evidence that conidia formation follows a circadian rhythm (Yarwood 1957). Conidial production and dispersal follow a diurnal cycle, with peak release occurring mid-

morning to afternoon. Some species of powdery mildew fungi form a single conidium on the conidiophores while others produce chains of conidia. The asexual cycle can repeat many times during the season and cause the disease to spread rapidly. In contrast, the sexual cycle typically occurs at the end of the growing season and produces survival structures which allow the pathogen to persist until the next growing season (Yarwood 1957).

In general, most powdery mildew fungi are heterothallic and undergo a sexual cycle. Sexual reproduction in heterothallic powdery mildew fungi requires fungal isolates with complementary mating types (Jarvis et al. 2002). Genes at the MAT1 locus determine mating type in powdery mildew fungi. The MAT1-1 and MAT1-2 idiomorphs are characterized by genes that encode an alpha 1 box and a high mobility group domain, respectively (Turgeon and Yoder 2000). The gene products function as pheromones that permit mating when both are present. In contrast, homothallic powdery mildew fungi possess both the MAT1-1 and MAT1-2 within the same cell, and consequently are universally compatible and capable of reproducing by haploid selfing (Billiard et al. 2011, 2012). Homothallism has been documented in the powdery mildew species, *Podosphaera plantaginis*, a pathogen of *Plantago lanceolata* (English plantain; Tollenaere and Laine 2013). Homothallism is considered rare in powdery mildew fungi, however, this may be due to a lack of research in the area.

The sexual structures of mature mycelium, known as gametangia, are characterized as a male antheridium and female ascogonium. Plasmogamy, the fusion of the gametangia, causes the nucleus to move from the antheridium to the

ascogonium and results in dikaryotization (Glawe 2008). Asci form from the dikaryotization event. Following conjugation, a survival structure known as the chasmothecia forms. This ascocarp has distinctive appendages and contains the asci and ascospores. Depending on the ecology of the host, chasmothecia remain firmly attached to the host substrate or disperse to other plant tissues (Gadoury et al. 2015). The overwintering survival period for powdery mildew fungi typically occurs from November through January in the northern hemisphere (Jarvis et al 2002).

*Podosphaera macularis* reproduces both sexually and asexually. The fungus is heterothallic and both mating types are required for sexual reproduction. The MAT1-1 and MAT1-2 mating types are found in approximately a one-to-one ratio in Europe and on wild hop plants in the eastern United States (Wolfenbarger et al. 2015). Only the MAT1-1 mating type of *P. macularis* has been reported in the Pacific Northwest hop growing region (Wolfenbarger et al. 2015; Gent et al. 2020). Thus, this powdery mildew fungus relies on asexual reproduction to perpetuate the disease in the Pacific Northwest region.

## Powdery Mildew Infection, Dispersal, Survival

**Infection.** The infection process of powdery mildews begins when ascospores or conidia land on a susceptible host plant and germinate (Hückelhoven 2005). It has been proposed that an enzymatic process initiates infection by overcoming the physical barrier of the host plant (Jarvis et al. 2002). A germ tube forms a specialized hyphal appressorium, which produces a penetration peg to enter the epidermal layers

of the host surface (Godwin et al. 1987). After penetration, a haustorium develops from the penetration peg. The haustorium absorbs nutrients from its host and maintains the parasitic relationship by releasing effectors that modulate host defense responses (Panstruga and Dodds 2009; Lo Presti and Kahmann 2017). Upon infection, plant pathogens release pathogen-associated molecular patterns (PAMPs) and effectors which can trigger a plant immune response (Rafiqi et al. 2012; Sarris et al. 2016). In studies comparing powdery mildew fungi on monocots and dicots, the effectors secreted by the pathogen were proposed to confer host specificity and virulence (Wu et al. 2018).

**Dispersal.** Wind dispersal is the primary mode of inoculum dissemination for powdery mildew fungi (Jarvis et al. 2002). Ascospores and conidia are wind dispersed and begin to germinate upon landing on host material. Moisture and turgor pressure differentials prompt the release of the ascospores from the chasmothecia after maturation. Conidia are proposed to be discharged though electrostatic charges or mechanical force (Jarvis et al. 2002). Conidia can be dispersed either singly or in chains and do not require water for germination. Moisture on leaves can impede conidial dispersal due to surface tension (Jarvis et al. 2002).

The environment is an important factor in the successful infection of powdery mildew fungi. In both the grape and hop powdery mildew, infectivity of conidia is reduced when ambient temperatures exceed 30°C (Delp 1954; Mahaffee et al. 2003b; Peetz 2009). In grape powdery mildew (*Erisiphe necator*), temperatures between 21-30°C are conducive for germination, infection, and growth (Delp 1954). The optimal

range for *P. macularis* infection and sporulation is between 18-27°C (Turechek et al. 2001). Cold induced resistance to powdery mildew of grape was demonstrated by exposing *Vitis vinifera* leaves to temperatures of between 2-8°C for 2 to 8 hours prior to infection (Moyer et al. 2016). Additionally, colony vigor was reduced when exposed to low temperatures. A similar response to cold temperatures was observed in hop powdery mildew (Weldon et al. 2017). The role of humidity has also been studied in relation to powdery mildew disease development, however, temperature was shown to play a more significant role in the germination and infection rate of conidia of *E. necator* (Delp 1954). Rain generally has a transitory negative impact on powdery mildew colonies (Sivapalan 1993; Mahaffee et al. 2003a; Thiessen et al. 2018). However, mycelium growth is restricted and infection efficiency declines on wet leaves as compared to dry (Jarvis et al. 2002).

**Survival.** Perennation is the ability for plant pathogens to persist over multiple seasons. Risk factors for pathogen perennation include favorable environmental conditions, the previous incidence of disease, and the quality of disease management (Caffi et al. 2013). Some plant pathogens have developed specialized structures that enable the pathogen to survive unfavorable conditions. Examples of these survival structures include oospores, chlamydospores, chasmothecia, and sclerotia. These survival structures often have double-celled, thick walls which prevent dehydration and damage to the spores.

As mentioned above, powdery mildew fungi are biotrophic and require living host tissue for reproduction. The cropping system and climate determines the mode of

survival for powdery mildews. In annual crops, there is a discontinuous supply of host material for the powdery mildew fungi in regions with hot, dry summers or cold, arid winters (Jarvis et al. 2002). Some powdery mildews can overwinter in leaf litter or on an alternate host. For example, powdery mildew of barley (*Blumaria graminus)* has been observed on other members of Poaceace in between plantings (Jarvis et al. 2002; Walker et al. 2011). Powdery mildew fungi can also persist throughout the year in subtropical or warmer temperate climates, or in a greenhouse setting.

In perennial cropping systems in temperate climates, powdery mildew fungi can additionally persist in dormant buds between cropping seasons (Jarvis et al. 2002). Successful bud perennation requires a susceptible cultivar, presence of powdery mildew, and environmental conditions conducive to infection. The phenology of woody perennial deciduous plants such as apple, grape, and hop allows their respective powdery mildew fungi to overwinter as mycelium in dormant buds. In grape, *E. necator* was found to persist as mycelium and chasmothecia within the host tissue, in addition to chasmothecia on the exfoliating bark (Grove and State 1987; Rumbolz and Gubler 2005). In apple, *Podosphaera leucotricha*, mycelium overwinters in vegetative buds and resumes growth at bud break in the spring (Jarvis et al. 2002). In hop, *P. macularis* can overwinter as chasmothecia on infested tissue and as mycelia on living tissue, mostly in dormant crown buds (Liyanage and Royle 1976). This overwintering of the mycelium within host tissues can lead to the development of heavily infected shoots in the spring following bud break (Jarvis et al. 2002). These infected shoots are termed flag shoots and exhibit a dense white mat of conidial chains covering portions of the young plant tissues. In regions where sexual

reproduction does not occur, flag shoots serve as the source of primary inoculum and contribute to the proliferation of the disease.

Chasmothecia are not reported consistently across regions where powdery mildews occur (Aslaf et al. 2013; Wolfenbarger et al. 2015). Temporal and geographic isolation mechanisms may contribute to the absence of both mating types or produce climates unfavorable for sexual reproduction or stimulation of ascocarp formation (Aslaf et al. 2013). Only the MAT1-1 mating type of *P. macularis* has been found in the Pacific Northwest region and in most commercial hop yards in the eastern U. S., therefore the fungus survives by overwintering in hop crown buds (Turechek et al. 2001; Wolfenbarger et al. 2015; Gent et al. 2019 and 2020). Flag shoots later emerge from infected buds during late February to early June (Gent et al. 2018). In the northern hemisphere, hop bud susceptibility to infection declines in early October with the onset of dormancy. Earlier powdery mildew infection during the prior growing season tends to increase the number of flag shoots in the subsequent production year. The probability of a flag shoot is rare and requires a relatively long period with high levels of inoculum in order for heavily-infected tissue to form (Gent et al. 2018). Flag shoots are found in low frequency in the Pacific Northwest, with less than 1% of susceptible hop plants being infected (Gent et al. 2008, 2018). A risk associated with the perennation of hop powdery mildew is that it could potentially lead to local adaptation of virulent races to specific cultivars. In the Pacific Northwest, widespread planting of the hop cultivars with R6-based resistance has selected for a new race of *P. macularis* that can grow on these previously resistant plants (Wolfenbarger et al. 2016).

## Powdery Mildew Management

Plant disease can have extensive impacts on crop yield and quality (Gaunt 1995). In general, interventions for disease management can take the form of planting resistant cultivars, cultural practices, and fungicide applications. The cropping system dictates which disease management approaches will be most effective. Powdery mildew fungi can perennate as mycelium or as chasmothecia in or on host tissue. Preventing perennation in annual cropping systems entails removing or burying infested plant debris between seasons and rotating with resistant hosts. Powdery mildew fungi are generally host specific, however, there are reports that powdery mildew of barley can persist on cereal stubble or other members of Poaceae (Jarvis et al. 2002; Walker et al. 2011). Removal of alternative hosts can be a means to curb powdery mildew spread through the following seasons. Planting cultivars resistant to powdery mildew also impedes the proliferation of disease. Cultivar rotation with diverse genetic backgrounds may reduce the opportunity for powdery mildew fungi to overcome host resistance (Wolfe 1985). If the powdery mildew fungus perennates in dormant buds of perennial crops, removal of diseased tissue or entire plants early in the season can prevent the spread of powdery mildew in perennial systems (Yarwood 1957; Jarvis et al. 2002). Disease management practices specific to hop are discussed below.

## Disease Resistance

In order to breed for resistant plants, one must first understand what qualities in a plant confer susceptibility. The susceptibility of plant tissue varies based on plant architecture and age. The specific architecture of leaf surface topography, caused by cutin formations and stomata shape, can affect the host plant's level of susceptibility to disease. Plant pathogens rely on cues from host tissue topography in order to successfully initiate the infection process (Walters 2006). Wynn (1976) demonstrated that the unique texture of the leaf surface of common bean (*Phaseolus vulgaris L*.) directed appressorium formation of the pathogen, *Uromyces appendiculatus*, the causal agent of bean rust (Wynn 1976). As a proof of concept, Hoch et al. (1987) used synthetic models of leaves to describe the behavior of germinating conidia and demonstrated the relationship between stomata height and necessary morphological precursors of successful infection. The majority of powdery mildew species have a very narrow host range, which also may depend on host topography as a cue for infection (Babu et al. 2002).

Age-related resistance (ARR) or ontogenic resistance refers to the resistance gained by the host as plant tissues mature (Hu and Yang 2019). This form of resistance can be adopted in agricultural systems as a disease management strategy. For example, planting date can be adjusted to avoid exposing susceptible plant tissues to a seasonally active pathogen (Hu and Yang 2019). While powdery mildew infections can occur on all parts of the plant, there is evidence of partial and complete ontogenic resistance developing as certain host tissues mature. In a study, Concord grapes were shown to exhibit moderate resistance on the leaves and fruit while the rachis were highly susceptible to powdery mildew (Gadoury et al. 2007). In hop,

ontogenic resistance in leaves (Turechek et al. 2001) and partial ontogenic resistance was observed in cones that had matured past stage II (Twomey et al. 2015). Research has been conducted to determine the underlying molecular mechanisms behind age-related resistance to powdery mildew in field crops such as barley (Torres et al. 2017). However, it is difficult to determine if host resistance is caused by an age-related innate immune response or the result of morphological and physiological changes due to environmental stresses. Although knowledge of the molecular mechanisms behind ontogenic resistance is limited, plant breeders have targeted ARR traits in breeding programs (Hu and Yang 2019).

Disease resistance is a major objective for many plant breeders (Miedaner 2016). Resistance can come in the form of major and minor resistance genes. One flaw with relying on major genes is that mutations can occur within the pathogen population that would enable the pathogen to overcome the host resistance and cause infection (McDonald and Linde 2002). To circumvent this, plant breeders may pyramid major resistance genes through the introgression of several forms of resistance. An alternative method is to select plants with partial resistance to disease through quantitative or minor genes. Identifying quantitative resistance and introgressing it into elite germplasm is a method that could delay a pathogen's ability to overcome the host resistance (Mundt 2014). However, it is difficult to discern which genes impart quantitative disease resistance and to select for quantitative resistance in juvenile plants.

Additionally, plant breeders have targeted susceptibility genes such as Mildew Locus O (MLO) (Jørgenson 1992). This family of susceptibility genes are conserved

across plant host species and can confer broad spectrum protection against all isolates of the fungal pathogen (Consonni et al. 2006). MLO genes code for membrane proteins that are involved with vesicle transport, and activation of certain MLO genes inhibits protection against papillae formation in fungi (Pessina et al. 2016). MLO-based resistance has been successfully deployed in barley breeding programs against powdery mildew, however, pleiotropic effects from the mutation has resulted in enhanced susceptibility to other pathogens due to necrosis from elevated cell-death response (Büschges et al. 1997; Ge et al. 2016). Despite increased susceptibility to certain other plant pathogens, MLO genes are a promising target for powdery mildew resistance in other crop species (Feechan et al. 2008).

Hop breeders in England have been selecting hop plants resistant to powdery mildew since the early 1900's (Salmon 1913; Neve 1986). Powdery mildew fungi are obligate plant pathogens and therefore, require a mechanism for maintaining the relationship with the host without causing severe damage. The gene-for-gene hypothesis of host-pathogen interactions describes a mechanism in which a major gene in the host confers resistance and the pathogen in turn may overcome the resistance with a corresponding virulence factor (Flor 1955). At this time, there are seven established major genes associated with hop powdery mildew resistance (Rb, R1, R2, R3 R4, R5, and R6) (Royle 1978; Darby 2013). A resistance study with the English cultivar Wye Target found that the deposition of lignified tissue around the haustorium and production of fungitoxic compounds contributes to R2 resistance of powdery mildew (Godwin et al. 1987). Efforts have been made to characterize the hop powdery mildew R gene sequences with the intent to be used for marker assisted

selection (Kozjak et al. 2009). Resistance or R-genes have been deployed in varieties with varying levels of success as pathogens can evolve to overcome resistance. There has also been work to discover potential minor genes responsible for resistance to hop powdery mildew (Henning et al. 2011).

At the onset of the powdery mildew outbreak in the mid 1990's, it was imperative for hop breeders in the Pacific Northwest to select resistant cultivars to quell the disease. Although the Pacific Northwest hop growing region only has asexually reproducing powdery mildew, the pathogen has been able to overcome the major resistance within a number of years once a particular host genotype is widely deployed across the landscape (Wolfenbarger et al. 2016; Gent et al. 2017). As it takes over a decade for a hop cultivar to be developed and released, breeders must be strategic in the deployment of novel forms of resistance. Broad deployment of major R gene resistance in crops can lead to populations of pathogens that can overcome said resistance (Wolfe and Schwarzbach 1978). Prior to 2012, the most prevalent races of *P. macularis* in the Pacific Northwest had virulence that overcame the R-genes, RB, R3, and R5. In 2012, approximately 25% of the hop acreage in the Pacific Northwest was planted to cultivars that possessed R6-based resistance (Wolfenbarger et al. 2016). After 2012, the population of *P. macularis* that became widespread can additionally overcome R4- and R6-based resistance (Wolfenbarger et al. 2016), herein termed V6. This V6 population of hop powdery mildew has since become endemic across the Pacific Northwest (Wolfenbarger et al. 2016). Recent genomic analysis of hop has described a putative locus for the R6 gene that possess several putative R-genes based on molecular motifs (Padgitt-Cobb et al. 2020). Previous

work characterizing V6-isolates in the Pacific Northwest found evidence of a fitness penalty of isolates that had overcome R6-based resistance associated with decreases in the number of colonies and an increase in the latent period (Wolfenbarger et al. 2016). The reduced fitness of V6-isolates suggests that there may still be utility in R6 resistance in hop as a QTL but should not be relied on for suppression of the disease in the long term. Accurate identification of V6-virulent isolates or other race constructs of the hop powdery mildew fungus would be valuable for the Pacific Northwest hop growing region as we could notify growers if certain cultivar fields are at a heightened risk for infection and knowing such information could subsequently influence management decisions.

**Chemical and Cultural Control**

Another form of disease management is the application of organic and synthetic fungicide treatments to combat powdery mildew. The Fungicide Resistance Action Committee (FRAC) classifies fungicides into categories known as FRAC codes based on mode of action of the active ingredients (Wade and Delp 1985). Some of the fungicides approved for powdery mildews include FRAC group 3 demethylation inhibitor (DMI) fungicides which target sterol biosynthesis and inhibit cell membrane formation (Herman and Stenzel 2020), and group 11 quinone outside inhibitors that target the mitochondrial cytochrome $bc_1$ complex (Hollomon and Wheeler 2002), among others (Nelson et al. 2015). Organic treatments for powdery mildews include powdered milk, oils, potassium bicarbonate, plant extracts, microbial extracts, sulfur formulations and water applications (Yarwood 1957; Medeiros et al.

2012). Biological controls such as *Bacillus* spp. and *Ampelomyces quisqualis*, a mycoparasite of powdery mildew fungi, can also be used to impede infections or as a part of integrative pest management (Jacobsen et al. 2004; Kiss 2003). In general, though, such products have limited efficacy (Nelson et al. 2015) and are not recommended as stand-alone programs. Spray programs should include fungicides with different modes of action to delay the development of fungicide resistance, and be part of an integrated pest management program.

At the time of the initial hop powdery mildew epidemic, the only chemical registered for hop with efficacy against powdery mildew was sulfur (Mahaffee et al. 2003a). Since then fungicide treatments such as myclobutanil, trifloxystrobin, and quinoxyfen have been registered to manage powdery mildew on hop (Gent et al. 2008; Nelson et al. 2015). Fungicide treatments are employed to prevent or reduce disease development on leaves as well as cones, and to prevent the build-up of chasmothecia in regions that contain both mating types. The percentage of disease control on cones is influenced by foliar disease levels and the fungicide mode-of action (Nelson et al. 2015). Meta-analysis by Nelson et al. (2015) found that fungicide treatments containing quinoxyfen were effective at controlling hop powdery mildew. Typically, 10-12 fungicide applications are required per season for managing powdery mildew on a highly susceptible hop cultivar. However, disease development depends more on when the first fungicide application is made, not on the total number of applications or interval lengths (Mahaffee et al. 2003a). Fungicide applications early in hop cone development paired with basal foliage removal have been shown to be an effective method for controlling powdery mildew disease (Gent

et al. 2016; Twomey et al. 2015). Fungicide treatments later in the season may not substantially improve yield or cone health (Gent et al. 2014), nor affect the levels of bud perennation (Gent et al. 2019). Implementing late season fungicide treatments may be less effective due to the development of partial ontogenic resistance to hop powdery mildew (Twomey et al. 2015).

Since the initial outbreak of hop powdery mildew in the Pacific Northwest, cultural practices such as crowning and the removal of basal foliage have been adopted in addition to fungicide programs to combat the disease (Gent et al. 2008, 2012, and 2016). In the presence of the ascigerious state, methods used to reduce primary inoculum include cleanup of infested debris post-harvest, pruning foliage, and mounding soil over hop crowns. Early season management of hop powdery mildew can be accomplished through pruning by mechanical, chemical, and flaming (propane) methods. Pruning quality is correlated with the subsequent level of disease severity (Gent et al. 2012, 2018). Excellent pruning quality is marked by complete removal of foliage, and has been shown to reduce the risk of powdery mildew outbreaks (Gent et al. 2012, 2018). Mechanical pruning has been shown to be more effective at reducing flag shoots because it removes crown buds more completely than the chemical or propane methods (Royle 1978; Gent et al. 2008, 2018). Early cultural management of hop powdery mildew has been shown to be more effective than later season treatments. Canopy management later in the season involves midseason basal foliage removal, which makes the micro-climate of the hop canopy less conducive to powdery mildew infection (Gent et al. 2016). Historically, basal

foliage has been removed by hand, however, herbicides or nitrogen fertilizer solutions are now commonly employed to strip the leaves (Neve 1991).

**Detection**

Early disease detection improves the efficacy of disease management programs through implementation of control measures (Gholson 1978). Current methods for disease detection of powdery mildew include scouting for disease and detection of airborne spores (Falacy et al. 2007; Mahaffee et al. 2016). Effective scouting can be constrained by numerous factors, including inconsistent appearance of disease year to year, inaccurate disease diagnosis, difficulty detecting low levels of disease, and limited amount of resources dedicated to disease management. Integration and implementation of modern technologies such as social media platforms, imaging software, and molecular techniques could mitigate some deficiencies of traditional scouting methods (Mahaffee et al. 2016; Knauer et al. 2017; Mueller et al. 2018).

In grape, spore traps were deployed to detect the presence of conidial inoculum in the air in order to guide focused disease management response for grape powdery mildew (Falacy et al. 2007). Polymerase chain reaction (PCR)-based assays have been developed in conjunction with the spore trap collection to differentiate *Erysiphe necator* from other powdery mildew species (Falacy et al. 2007; Thiessen et al. 2016). Integration of molecular-based technologies in disease management programs would improve the efficiency of response through accurate pathogen identification and subsequent use of appropriate disease treatment.

**Assay Development**

Advancements in molecular techniques have allowed for the development of rapid diagnostic tests to determine the causal disease agent. Molecular assays rely on the differences in genetic sequences and variation in levels of gene expression (Ward et al. 2004). Some methods for differentiating between sequences include single nucleotide polymorphisms (SNPs) and presence/absence markers for unique loci (Ward et al. 2004). PCR-based assays are an efficient method to detect and differentiate between different plant pathogens with the incorporation of SNPs into primer and probe design. PCR and variants of the methods are used routinely in diagnostic assays for detection, differentiation of strains, and for predicting phenotype (Schaad and Frederick 2002; McCartney et al. 2003; Ward et al. 2004).

Numerous modifications of PCR have been developed to increase sensitivity of assays, reduce time, and enable field-based application (Cha and Thilly 1993; Ward et al. 2004). Incorporation of novel oligonucleotides such as locked nucleic acids (LNA) can be used to improve the fidelity of an assay through the increase of probe melting temperature. Locked nucleic acids are synthetically derived nucleic acid analogs that were developed to increase specificity in single strand DNA and RNA detection. LNA bases have a bicyclic structure created by a synthesized 2’-O, 4’-C methylene bridge (Koshkin et al. 1998). The reinforced structure of the LNA is useful for differentiating between single SNPs.

Various PCR-based methods have been developed for the detection and differentiation of plant pathogens and implemented for disease diagnosis and

epidemiological studies (Schaad and Frederick 2002). For example, Wolfenbarger et al. (2015) developed a traditional PCR-based assay to differentiate mating idiomorphs of *P. macularis*. This assay was used to assist in the identification of the mating type of *P. macularis* isolates found in the Pacific Northwest and provided valuable information about the population structure of hop powdery mildew in the region. Real-time PCR reactions have the advantage of multiplexing fluorescently-labeled detection probes in order to quantify the presence of multiple targets present in a sample (Wittwer et al. 2001; Schaad and Frederick 2002). A real-time PCR assay was developed using LNA to detect the presence of downy mildew on cucumber (*Pseudoperonospora cubensis*) and hop (*Pseudoperonospora humuli*) (Summers et al. 2015). Molecular-based assays that include LNA probes can serve as important tools for the detection and differentiation of plant pathogens in a field-based or lab setting. A rapid detection method for plant pathogen race determination could aid growers in disease management strategies and assist plant breeders in resistant germplasm selection (Hansen et al. 2016).

V6-isolates of the hop powdery mildew fungus have become endemic in the Pacific Northwest region since 2012. These isolates can persist on hop cultivars that were susceptible prior to 2012, in addition to those cultivars that possess R6-based resistance. This means that powdery mildew found on previously susceptible hop cultivars must undergo screening on a differential set of cultivars in order to determine which race is present. Race determination is an important part of disease diagnosis as it guides the appropriate disease management response. Knowledge of what races of *P. macularis* are present in a region or a particular field could inform

hop growers if their susceptible yards are at an increased risk for infection, enabling preemptive action. Additionally, a rapid detection method for race determination of the hop powdery mildew fungus could aid plant breeders with race-specific isolate selection for resistance screening of hop germplasm. Epidemiological studies would also benefit from hop powdery mildew race identification in determining the primary source of virulent races and track the spread of disease through a region.

**Conclusion**

This research aims to develop a rapid molecular assay to detect V6-isolates of the hop powdery mildew fungus. Current methods for determining virulence in *P. macularis* employ traditional inoculation of a set of differential hop cultivars. This method is costly, labor intensive, and can take up to three weeks to obtain results, assuming plant material is available. A molecular assay would bypass the need for maintenance of plant material and provide growers with results within hours. Overall, the goal of this project is to develop a diagnostic tool to aid researchers in the public and private sectors to inform hop growers on disease hazard associated with V6-races of *P. macularis*. Chapter 2 describes the methods used to develop the assay and discusses its application and utility.

# CHAPTER 2:

## Development of Diagnostic Assays for Race Differentiation of *Podosphaera macularis*

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Plant Disease

*In progress*

**Abstract**

Hop powdery mildew (caused by *Podosphaera macularis*) was confirmed in the Pacific Northwest in 1996. Before 2012, the most common race of *P. macularis* was able to infect plants that possessed powdery mildew resistance based on the R-genes Rb, R3, and R5. After 2012, two additional races of *P. macularis* were discovered that can overcome the resistance gene R6 and the partial resistance found in the cultivar Cascade. These three races now occur throughout the region, which can complicate management and research efforts because of uncertainty on which race(s) may be present in the region and able to infect susceptible hop genotypes. Current methods for determining the races of *P. macularis* are labor intensive, costly, and typically require more than 14 days to obtain results. We sought to develop a molecular assay to differentiate races of the fungus possessing virulence on plants with R6, referred to as V6-virulent, from other races. The transcriptomes of 46 isolates of *P. macularis* were sequenced to identify loci and variants unique to V6-isolates. Fourteen primer pairs were designed for 10 candidate loci that contained single nucleotide polymorphisms (SNP) and short insertion-deletion mutations. Two differentially-labeled locked nucleic acid probes were designed for a contig that contained a conserved SNP associated with V6-virulence. The resulting multiplexed real-time PCR assay was validated against 46 V6 and 54 non-V6 *P. macularis* isolates collected from the United States and Europe. The assay had perfect discrimination of V6-virulence among isolates of *P. macularis* originating from the western U.S. but failed to predict V6-virulence in three isolates collected from

Europe. The specificity of the assay was tested with different species of powdery mildew fungi and other microorganisms associated with hop. Weak non-specific amplification occurred with powdery mildew fungi collected from *Vitis vinifera*, *Fragaria* sp., and *Zinnia s*p.; however, non-specification amplification is not a concern when differentiating pathogen race from colonies on hop. The assay has practical applications in hop breeding, epidemiological studies, and other settings where rapid confirmation of pathogen race is needed.

## Introduction

The cultivated hop, *Humulus lupulus* L., is a dioecious, perennial climbing bine with annual shoots (Neve 1991). Hop has been grown for centuries for its female inflorescence known as a cone. The lupulin glands of hop cones contain aromatic compounds that impart bitterness along with unique aromas and flavors to beer. Hop plants are commercially grown in regions between 35 to 55 latitudes north and south due to the daylight requirements for flower development (Burgess 1964). The states of Idaho, Oregon, and Washington fall within the optimum growing range for hop and produce 98% of the hops harvested in the United States (USDA NASS 2019). The demands of the brewing industry dictate which cultivars are planted (Haunold 1981), leading to an increased risk of disease susceptibility due to the preference for brewing quality over disease resistance in most instances (Neve 1991).

Hop powdery mildew, caused by the fungus, *Podosphaera macularis*, is one of the most destructive diseases of hop. Decreases in photosynthetic activity from

relatively small amounts of the disease are believed to have little impact on the hop plant due to the extensive size of the hop canopy. However, infection of the female inflorescence can have devastating effects on crop production by damaging the hop cones to the extent that they are no longer marketable (Gent et al. 2014; Gent et al. 2018). Hop powdery mildew can be managed through cultural methods or chemical interventions. However, the most cost-effective way for producers to prevent the proliferation of the pathogen is through the deployment of disease resistant cultivars. Hop plants are clonally propagated; so consequently, the cultivars used are genetically uniform within a field. Given the genetic uniformity and intense disease pressure, resistance has not proven to be durable in commercial production (Royle 1978; Wolfenbarger et al. 2016; Gent et al. 2017).

In 1997, hop powdery mildew was first observed at damaging levels in commercial hop fields in the Pacific Northwest (Ocamb et al. 1999). In 1998, powdery mildew was documented in the hop growing regions of Oregon and Idaho. By 1999, the majority of hop acreage in Washington was affected by the disease (Ocamb et al. 1999). Growers were unprepared for the powdery mildew outbreak and experienced severe losses as a result. In the Pacific Northwest region, costs for disease management and yield losses due to hop powdery mildew in 1999 and 2000 were estimated to be 15% of the total crop revenue (Turechek et al. 2001).

At the onset of the powdery mildew outbreak in the mid 1990's, it became imperative for hop breeders in the Pacific Northwest to select resistant cultivars to quell the disease, a breeding objective that had been pursued in international breeding

programs since the early 1900's (Salmon 1917; Neve 1986). Known resistance genes to hop powdery mildew have been reported and designated as Rb, R2, R3, R4, R5, and R6 (Royle 1978; Darby 2013). Prior to 2012, the most common race of *P. macularis* found in the Pacific Northwest was virulent on plants possessing the R-genes Rb, R3, and R5 (Gent et al. 2017). Widespread planting of cultivars with R6-based resistance lead to the occurrence of new races of powdery mildew capable of overcoming resistance gene R6, termed V6 isolates (Wolfenbarger et al. 2016; Gent et al. 2017). Previous work characterizing V6-isolates in the Pacific Northwest found evidence of a fitness penalty of isolates that had overcome R6-based resistance, expressed as a decrease in the average number of colonies per leaf and an increase in the latent period (Wolfenbarger et al. 2016). The reduced fitness of V6-isolates suggests that there may still be utility in R6 resistance in hop as a quantitative trait locus (QTL) but this gene should not be relied on alone for suppression of the disease.

V6 isolates of the hop powdery mildew fungus have become endemic in the Pacific Northwest region since 2012 (Wolfenbarger et al. 2016). These isolates can persist on hop cultivars that were susceptible prior to 2012, in addition to those cultivars that possess R6-based resistance. This means that powdery mildew found on previously susceptible hop cultivars must undergo screening on a differential set of cultivars in order to determine which race is present. Race determination is an important part of disease diagnosis as it guides the appropriate disease management response. Knowledge of what races of *P. macularis* are present in a region or a particular field could inform hop growers if their susceptible yards are at an increased

risk for infection, enabling preemptive action. Additionally, a rapid detection method for race determination of the hop powdery mildew fungus could aid plant breeders with race specific isolate selection for resistance screening of hop germplasm. Epidemiological studies would also benefit from hop powdery mildew race identification in that it could assist in determining the primary source of virulent races and tracking the spread of disease through a region.

This research aimed to develop a rapid molecular assay to detect V6-virulent isolates of the hop powdery mildew fungus in the Pacific Northwest. A molecular assay would bypass the need for maintenance of a wide array of plant material and provide growers with results within hours rather than weeks.

## Materials and Methods

*Plant materials*. Hop plants were propagated from softwood cuttings and maintained in a greenhouse free of powdery mildew by regular atomizing of sulfur. The powdery mildew susceptible cultivar Symphony and the R6-cultivar Nugget were grown for isolate maintenance and V6-virulence identification, respectively. Plants were grown in Metro-Mix 840 (Sun Gro Horticulture, Hubbard, Oregon) for approximately 14 to 21 days and were watered daily, receiving Peter's Professional 20-20-20 fertilizer (Sun Gro Horticulture) at each irrigation. The greenhouse was maintained at 20 to 25ºC with a 14-h photoperiod. Isolates of *P. macularis* were maintained on detached hop leaves (Wolfenbarger et al. 2016). Briefly, young, unfurled leaves from the top

two nodes were surface disinfested with 70% ethanol and rinsed for 30 seconds with water and dried. Disinfested leaves were detached and placed in a double Petri dish (Pearson and Gadoury 1987) with water in the lower petri dish and inoculated with isolates. *P. macularis* isolates were transferred onto fresh leaves every 2 to 3 weeks.

*DNA sample preparation.* Conidia and hyphae were collected from *P. macularis* colonies 2 to 3 weeks after inoculation. DNA was extracted from powdery mildew isolates using a Chelex extraction procedure (Brewer and Milgroom 2010). Extracted DNA was quantified using the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 1 ng/µL.

*Race characterization of* P. macularis *isolates*. During 2019, 40 samples of leaves or cones with powdery mildew were collected from commercial hop yards in Oregon (21 isolates) and Washington (19 isolates) (Table 1). Isolates were obtained by bulk transfer of *P. macularis* (i.e., not reduced to a single conidial chain) onto detached leaves of cv. Symphony or Nugget as described by Wolfenbarger et al. (2016). Isolates were maintained through successive transfers onto Symphony, with routine transfers on Nugget to ensure selection for V6-virulence. Additionally, DNA was obtained from 60 isolates of *P. macularis* previously characterized for V6-virulence on cv. Nugget (Wolfenbarger et al. 2016). These were 25 isolates with confirmed V6-

virulence and 35 lacking V6-virulence (Table 1). DNA was extracted as described previously and stored at -20°C until use in PCR assays as described below.

*PCR primer design and synthesis.* Gent et al. (2020) previously described identification of genetic variants in the transcriptome of isolates of *P. macularis* that are associated with V6-virulence in the fungal population in the Pacific Northwestern region of United States. In the present study, we examined a subset of the 16 loci reported by Gent et al. (2020) for their utility as a target in an allele-specific PCR for differentiation of V6-virulence (Table 2). Primers were designed to flank single SNPs present in the highly differentiated loci and were sequence verified. Primers were designed using Geneious version R11.1 ( https://www.geneious.com ) and the IDT OligoAnalyzer (https://www.idtdna.com/calc/analyzer) to be approximately 20 nucleotides in length with a Tm of 60°C. Isolates collected from Oregon and Washington with confirmed V6-virulence (HPM-609 and HPM-666) or lacking V6-virulence (HPM-663 and HPM-956) were used as positive and negative controls during the design process.

The primers were used in PCR assays carried out in 20-µl volumes containing 10 µl of 2× Prime Time Gene Expression Master Mix (1× final concentration; Integrated DNA Technologies (IDT), Coralville, IA, USA), 500 nM of each forward and reverse primer, and 2 ng of template DNA. PCR was conducted using a Bio-Rad C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA) with the following reaction conditions: initial denaturation of DNA at 95°C for 2 min, followed by 40

cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 seconds. Reactions were held at 72°C for 10 min for the final extension. The ITS region was also amplified using ITS primers ITS1 and ITS4 (White et al. 1990) as a further control to ensure amplification of the template DNA was possible. Primers were ordered through IDT. PCR products were verified by gel electrophoresis prior to bidirectional sequencing by Eurofins Genomics (Louisville, KY, USA). Sequences of each region were aligned using Geneious to verify the presence of each diagnostic SNP. Primers that produced bright bands in conventional PCR reactions and were sequence-verified were selected to use in probe design, as detailed below.

*LNA probe and assay design, optimization, and testing.* Locked nucleic acid (LNA) probes and qPCR assays were designed to differentiate isolates of *P. macularis* from the Pacific Northwest that possess SNPs associated with V6-virulence and the non-V6-virulent wild-type phenotype (Table 3). Assays were designed to meet the requirements of the minimum information for publication of quantitative real-time experiments (Bustin et al. 2009). PCR was conducted using a Bio-Rad CFX96 C1000 Touch thermal cycler (Bio-Rad). Samples were run in Hard-Shell® 96-well plates and sealed with Microseal 'B' seals (Bio-Rad). Each reaction consisted of 10 µl of iTaq (1 × final concentration; Bio-Rad), 500 nM forward and reverse primers (IDT), 250 nM of each LNA probe (IDT), and 2 ng of template DNA in a final volume of 20 µL. Reactions were held at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 70°C for 30 s, with fluorescence measured after each 70°C step. A no template

control was included in each run. Cycle threshold (Cq) values were determined using Bio-Rad CFX Manager software version 3.0. Positive probe detection was defined as amplification occurring ≤ 38 Cq with greater than 2,000 relative fluorescence units (RFU).

LNA reactions were run as previously described at varying annealing temperatures to optimize the reaction for race-specific differentiation. Six annealing temperatures were tested, ranging from 66 to 76°C. In each test of optimal annealing temperature, two isolates of each race were tested, along with a negative control which lacked DNA template. Fungal isolates collected from Oregon and Washington with confirmed V6- virulence (HPM-609 and HPM-666) and lacking V6-virulence (HPM-663 and HPM-956) were used as positive and negative controls, respectively, during the design process. LNA reactions were performed using a Bio-Rad CFX96 C1000 Touch thermal cycler and the following reaction conditions: initial denaturation of 95.0°C for 3 min, followed by 39 cycles of 95.0°C for 30 s and 66°C (or varied temperatures during optimization tests) for 30 s, with fluorescence measured after each annealing and extension step. PCR reagents and conditions were as described previously. Each qPCR run included a negative control which lacked DNA template. Cq values were measured and positive reactions were defined as described previously.

Following reaction optimization, LNA probes and assays were tested for sensitivity and specificity to V6 isolates of *P. macularis*. Assay sensitivity was determined by running 10-fold dilutions of *P. macularis* DNA ranging from 2,000 to

2 pg/reaction. Two V6-virulent isolates were tested (isolate HPM-609 and isolate HPM-666) along with two non-V6 isolates (HPM-663 and HPM-956). The limit of detection was defined as the lowest concentration of template DNA that still resulted in detection by the target probe, as previously defined. Standard curves for each probe were generated using Bio-Rad CFX Manager software version 3.0. Mixed-isolate reactions were also conducted at varying DNA concentrations to determine the effect on each LNA probe to differentiate the races in a mixed sample. Two race combinations were tested (V6-virulent isolate HPM-1220 and non-V6 isolate HPM-1040), at each of eight DNA concentrations (Table 4). Reaction mixes were prepared as previously described, with the exception of 4µl of template DNA (2µl of HPM-1220 and 2µl of non-V6 HPM-1040 DNA, each of variable concentration) being added to each reaction well.

Assay specificity to V6-isolates was tested with 54 non-V6-isolates and 46 V6-isolates (Table 5). Each reaction consisted of 10 µl of iTaq (1× final concentration; Bio-Rad), 500 nM forward and reverse primers, 250 nM of each LNA probe, and 2 µL of template DNA brought to a final volume of 20 µL. Reactions were held at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 70°C for 30 s, with fluorescence measured after each 70°C step. The potential for amplification of nontarget organisms also was tested with the above PCR conditions. DNA was extracted using the Chelex method from powdery mildew fungi samples that were collected from different host species found in the Pacific Northwest (Table 6). DNA was extracted using the FastDNA SPIN Kit (MP Biomedicals, Solon, OH, USA) from other organisms associated with hop (*Alternaria alternata, Botrytis*

*cinerea, Diplodia seriata, Pseudoperonospora humuli, Fusarium sambucinum, Lecanicillium attenuatum, Lecanicillium lecanii, Phomopsis tuberivora, Phomopsis* sp., *Verticillium nonalfalfae*) that had previously been collected and preserved. DNA from hop plant tissue was extracted using DNeasy Plant Mini Kit (Qiagen) and was tested for cross reaction. A negative control lacking template was included in each qPCR run. Cq values were measured and positive reactions were defined as described previously.

## Results

*PCR primer design and synthesis.* Fourteen primer pairs were designed from contigs that possess variants that differentiate V6 from non-V6 isolates based on the *G*' statistic (Gent et al. 2020). No amplification occurred with primers designed from contigs 674, 1456, 3456, and 5050. Contig 2251 and 2407 yielded single amplicons in conventional PCR assays and contained SNPs at the predicted locations following sequence verification. BLASTX results for contig 2407 showed sequence similarity to a mitochondrial import inner membrane translocase protein while there were numerous (integrase, reverse transcriptase, retro virus-related Pol polyprotein) different BLASTX results for contig 2251. Therefore, we selected contig 2407 for probe and qPCR assay development due to the consistency of the BLAST results.

*LNA probe and assay design, optimization, and testing.* Two differentially-labeled LNA probes, N2407.WT and N2407.P1, were designed to target a region of contig 2407 and differentiate V6-isolates from non-V6 isolates of *P. macularis* from the Pacific Northwest. At the optimized annealing temperature of 70°C, the LNA probes differentiated 43 V6-isolates from 54 non-V6 isolates derived from the Pacific Northwest (Table 5). Perfect discrimination was observed between Pacific Northwest-derived V6-isolates, detected by the FAM fluorophore in N2407.P1 probe, and non-V6-isolates, detected by the HEX fluorophore in the N2407.WT probe (Table 5). Cq values for the Pacific Northwest V6-isolates ranged from 28.10 to 37.43 (mean 33.25) with the FAM fluorophore. Pacific Northwest V6-isolates did not generate Cq values below the 38 Cq threshold with the HEX fluorophore. However, the V6-isolates from Germany were detected by the HEX fluorophore with Cq values ranging from 28.94 to 35.08 (mean 31.59). Non-V6 isolates generated Cq values of 27.66 to 36.83 (mean 32.11) with the HEX fluorophore but did not generate Cq values below the 38 Cq threshold with the FAM fluorophore. Negative controls lacking template failed to produce amplification in any LNA qPCR assays.

In each of the three biological replicates, 20 pg was the minimum amount of DNA that could be detected by each LNA probe (Table 4). When DNA concentrations of V6 and non-V6 isolates were equal, the LNA assay performed similarly whether running a two isolate mixture of differing race or a reaction with only a single isolate (Table 4). During a single-race reaction, either the HEX or FAM fluorophore was detected per well, depending on the race of the template DNA added

to that well. During the mixed-race reaction, both the FAM and HEX fluorophores were detected in each well. Cq values were comparable with those observed during the single-race limit of detection reactions (Table 4). The assay was able to detect DNA from both samples even when the DNA sample concentrations within the mixed race sample differed by more than one order of magnitude.

When the multiplex assay was tested with DNA samples from other powdery mildew fungi, amplification occurred with all powdery mildews except those isolated from *Eucalyptus*. Samples taken from *Plantago major* had amplification with both probes with values below the 38 Cq threshold but with RFU values around 1,500. Powdery mildew collected from *Vitis vinifera, Zinnia sp.*, and *Fragaria sp.* had non-specific amplification with both probes with Cq values at or below the 38 Cq threshold (Table 6). The rest of the samples had weak amplification curves with less than 1500 RFU and were regarded as potential background noise. DNA from other hop associated organisms and the hop plant tissue did not amplify (data not shown).

**Discussion**

The goal of this project was to develop a molecular diagnostic assay capable of distinguishing V6-isolates of *P. macularis* from other races of the hop powdery mildew fungus. At this time, V6 isolates of *P. macularis* have been predominately found in the Pacific Northwestern region of the United States where there has been broad deployment of hop cultivars containing R6-based resistance (Wolfenbarger et al. 2016). Quick and accurate race detection of plant pathogens, especially of

powdery mildew fungi, can aid hop growers in disease management decisions as well as provide important information in epidemiological studies involving population structure dynamics and pathogen dispersal. This assay could offer a more rapid and accessible alternative to race detection than methods currently used for characterizing *P. macularis* that generally require multiple weeks for definitive results (Wolfenbarger et al. 2016; Gent et al. 2017).

After analyzing the transcriptomes of 46 isolates of *P. macularis* collected in the Pacific Northwest, 16 candidate loci were identified that differentiate V6 isolates from the other two races widely prevalent in this region. Fourteen primer pairs were designed from these distinctive loci and tested with preliminary conventional PCR screening with four Pacific Northwestern *P. macularis* isolates with known virulence. We selected two contigs (contigs 2251 and 2407) that yielded single amplicons in conventional PCR assays and contained SNPs at the predicted locations following sequence verification.

The assay was population-specific to *P. macularis* isolates collected from hop plants in the Pacific Northwestern region of the U.S. The assay did not predict V6-virulence in three isolates collected from Germany. Contig 2407 used for assay development contains a SNP that is associated with V6-virulence in the Pacific Northwest, but this SNP is not necessarily a causal mutation that confers virulence to the pathogen. As this assay was designed from isolates collected solely from the Pacific Northwest, it is not unexpected that V6-isolates originating from other countries were not detected. The mutation that confers V6-virulence to *P. macularis*

isolates originating from the Pacific Northwestern U.S. likely originated from an extant isolate in this region (Gent et al. 2020) that subsequently became widespread following a widespread planting of R6-based resistant hop varieties in commercial fields. Multiple mutations or other mechanisms could lead to similar phenotypes in different populations.

The assay we designed is constrained by the amount of fungal DNA needed to detect the presence of *P. macularis*, with the limit of detection approximately 20 pg. Therefore, this assay would not be appropriate to use in applications such as environmental sampling that requires sensitivity near the single conidium level (Gent et al. 2009; Mahaffee and Stoll 2016; Thiessen et al. 2016). Rather, this assay is appropriate for determining pathogen race from colonies growing on hop tissue. The amount of DNA extracted from one colony (approximately 0.5 cm diameter in size) of *P. macularis* is approximately 730 pg, which would more than suffice for the race determination of samples collected from a hop yard.

The differentially-labeled probes can detect the presence of both V6 and non-V6 isolates simultaneously, which would be helpful in rapidly determining pathogen race when powdery mildew occurs on hop cultivars that do not possess R6-based resistance. This would not have been feasible without considerable effort using traditional inoculation-based race screening methods. Determining whether mixed infections are present requires inoculations onto multiple cultivars, a differential cultivar with R6 and also a susceptible cultivar that permits growth of non-V6-virulent isolates. Colonies that form on the later would then need to purified and re-

inoculated to isolate individuals that lack V6-virulence. This tedious process is not amenable to the scaling up required in a research context where hundreds of samples must be processed.

Since 2012, the prevalence of V6 isolates of *P. macularis* has increased throughout the primary hop growing areas in the western U.S. (Wolfenbarger et al. 2016). Our assay has utility in epidemiological studies to quantify regional differences in prevalence of V6 isolates on certain cultivars. Wolfenbarger et al. (2016) previously observed regional differences in the prevalence of *P. macularis* isolates with V6-virulence on hop cultivars lacking R6 depending on overall disease severity in a region. Our assay could facilitate further investigation of factors influencing propagule density of V6-virulent isolates on certain cultivars.

Aggressiveness of certain isolates may depend on the cultivar (Wolfenbarger et al. 2016). This has implications for hop breeding programs in that the assay could be used to identify pathogen race on germplasm and could lead to more directed selection with cultivars with R6-based resistance as parental stock. This assay can also be applied as a rapid diagnostic tool to detect and confirm the spread of V6- isolates from the Pacific Northwest to other regions on planting material, which appears to occur within and between production regions (Gent et al. 2020). Further, the primers developed for this assay have been adapted for an amplicon sequencing platform to massively scale genotyping (Weldon et al. 2020).

While the assay can accurately detect and differentiate between V6 and non-V6 isolates of *P. macularis* derived from the Pacific Northwest, the assay does not

differentiate between the pre-2012 and ‘Cascade’-adapted races of the fungus. The pre-2012 and ‘Cascade’-adapted races of the fungus may infect plants possessing Rb, R3, and R5, with the latter additionally overcoming the partial resistance in Cascade (Gent et al. 2017). There would be utility in further differentiation and identification of the three known races of *P. macularis* known to occur in the Pacific Northwest for the above-mentioned reasons. Future work will involve analyzing transcriptome variants for the development of an assay specific to the ‘Cascade’-adapted race of *P. macularis*.

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**Table 1.** *Podosphaera macularis* isolates previously collected

| Isolate | Collection Year | State or Country[a] | Original Cultivar | Race[b] |
|---|---|---|---|---|
| HPM-198 | 1999 | Germany | Northern Brewer | V6 |
| HPM-199 | 1999 | Germany | Northern Brewer | Non-V6 |
| HPM-200 | 2000 | Germany | Northern Brewer | Non-V6 |
| HPM-201 | 1999 | Germany | Northern Brewer | Non-V6 |
| HPM-202 | 2008 | Germany | Northern Brewer | V6 |
| HPM-203 | 1999 | France | Northern Brewer | Non-V6 |
| HPM-204 | 2004 | England | Northern Brewer | Non-V6 |
| HPM-205 | 1999 | Germany | Northern Brewer | V6 |
| HPM-258 | 2012 | WA | Nugget | V6 |
| HPM-368 | 2012 | ID | Apollo | V6 |
| HPM-374 | 2012 | ID | Apollo | V6 |
| HPM-609 | 2013 | OR | Nugget | V6 |
| HPM-663 | 2014 | OR | Magnum | Non-V6 |
| HPM-666 | 2014 | WA | Apollo | V6 |
| HPM-697 | 2014 | WA | Cascade | Non-V6 |
| HPM-704 | 2014 | OR | Nugget | V6 |
| HPM-712 | 2014 | OR | Nugget | V6 |
| HPM-713 | 2014 | OR | Nugget | V6 |
| HPM-714 | 2014 | OR | Nugget | V6 |
| HPM-715 | 2014 | OR | Nugget | V6 |
| HPM-716 | 2014 | OR | Nugget | V6 |
| HPM-718 | 2014 | OR | Nugget | V6 |
| HPM-719 | 2014 | OR | Nugget | V6 |
| HPM-724 | 2014 | OR | Nugget | V6 |
| HPM-726 | 2014 | OR | Nugget | V6 |
| HPM-956 | 2016 | WA | Cascade | Non-V6 |
| HPM-967 | 2016 | WA | Cascade | Non-V6 |
| HPM-1040 | 2016 | WA | Cascade | Non-V6 |
| HPM-1053 | 2016 | WA | CTZ | Non-V6 |
| HPM-1055 | 2016 | WA | Chinook | Non-V6 |
| HPM-1056 | 2016 | WA | CTZ | Non-V6 |
| HPM-1059 | 2016 | WA | Cascade | Non-V6 |
| HPM-1064 | 2016 | WA | Cluster | Non-V6 |
| HPM-1066 | 2016 | WA | Simcoe | Non-V6 |
| HPM-1068 | 2016 | WA | Nugget | V6 |
| HPM-1075 | 2016 | WA | Bravo | V6 |
| HPM-1077 | 2016 | WA | Nugget | V6 |
| HPM-1078 | 2016 | OR | Cascade | Non-V6 |
| HPM-1081 | 2016 | OR | Cascade | Non-V6 |
| HPM-1084 | 2016 | OR | Cascade | Non-V6 |
| HPM-1099 | 2016 | NY | Zeus | Non-V6 |

| | | | | |
|---|---|---|---|---|
| HPM-1101 | 2016 | MN | Wild hop | Non-V6 |
| HPM-1107 | 2016 | MN | Wild hop | Non-V6 |
| HPM-1112 | 2016 | OR | Apollo | V6 |
| HPM-1120 | 2016 | OR | Nugget | V6 |
| HPM-1122 | 2016 | OR | Nugget | V6 |
| HPM-1123 | 2016 | OR | Nugget | V6 |
| HPM-1124 | 2016 | WA | Ekuanot | Non-V6 |
| HPM-1126 | 2016 | WA | Cascade | Non-V6 |
| HPM-1137 | 2016 | MD | Wild hop | Non-V6 |
| HPM-1151 | 2016 | MN | Fuggle 4n seedling | Non-V6 |
| HPM-1152 | 2016 | MN | Rulo-E seedling | Non-V6 |
| HPM-1182 | 2017 | WI | Galena | Non-V6 |
| HPM-1183 | 2017 | WI | Southern Cross | Non-V6 |
| HPM-1190 | 2017 | England | Feral female seedling | Non-V6 |
| HPM-1193 | 2017 | England | Feral female seedling | Non-V6 |
| HPM-1196 | 2017 | England | Experimental genotype | Non-V6 |
| HPM-1198 | 2017 | England | Experimental genotype | Non-V6 |
| HPM-1200 | 2017 | England | Feral female seedling | Non-V6 |
| HPM-1203 | 2017 | England | Seedlings PG genotypes | Non-V6 |
| HPM-1216 | 2019 | OR | Nugget | V6 |
| HPM-1217 | 2019 | OR | Nugget | V6 |
| HPM-1218 | 2019 | OR | Nugget | V6 |
| HPM-1220 | 2019 | WA | Bravo | V6 |
| HPM-1221 | 2019 | WA | Bravo | V6 |
| HPM-1222 | 2019 | WA | Bravo | V6 |
| HPM-1223 | 2019 | OR | Strata | Non-V6 |
| HPM-1224 | 2019 | OR | Strata | Non-V6 |
| HPM-1226 | 2019 | OR | Nugget | V6 |
| HPM-1227 | 2019 | OR | Nugget | V6 |
| HPM-1229 | 2019 | OR | Nugget | V6 |
| HPM-1230 | 2019 | OR | Nugget | V6 |
| HPM-1231 | 2019 | WA | Eureka | V6 |
| HPM-1232 | 2019 | WA | Eureka | V6 |
| HPM-1233 | 2019 | WA | Citra | Non-V6 |
| HPM-1234 | 2019 | WA | Citra | Non-V6 |
| HPM-1235 | 2019 | WA | Citra | Non-V6 |
| HPM-1236 | 2019 | WA | Citra | Non-V6 |
| HPM-1237 | 2019 | WA | Citra | Non-V6 |
| HPM-1238 | 2019 | WA | Citra | Non-V6 |
| HPM-1239 | 2019 | OR | Citra | Non-V6 |
| HPM-1240 | 2019 | OR | Citra | Non-V6 |
| HPM-1241 | 2019 | OR | Citra | Non-V6 |

| | | | | |
|---|---|---|---|---|
| HPM-1242 | 2019 | OR | Citra | V6 |
| HPM-1243 | 2019 | OR | Citra | V6 |
| HPM-1244 | 2019 | OR | Citra | V6 |
| HPM-1251 | 2019 | OR | Citra | V6 |
| HPM-1252 | 2019 | WA | Citra | Non-V6 |
| HPM-1253 | 2019 | WA | Citra | Non-V6 |
| HPM-1254 | 2019 | WA | Citra | Non-V6 |
| HPM-1256 | 2019 | WA | Citra | Non-V6 |
| HPM-1257 | 2019 | WA | Citra | Non-V6 |
| HPM-1258 | 2019 | WA | Citra | Non-V6 |
| HPM-1260 | 2019 | WA | Citra | Non-V6 |
| HPM-1261 | 2019 | WA | Citra | Non-V6 |
| HPM-1263 | 2019 | OR | Citra | V6 |
| HPM-1264 | 2019 | OR | Citra | V6 |
| HPM-1265 | 2019 | OR | Citra | V6 |
| HPM-1266 | 2019 | OR | Citra | V6 |
| HPM-1267 | 2019 | OR | Citra | V6 |

[a] OR= Oregon, WA= Washington, ID = Idaho, NY = New York, MN = Minnesota, MD = Maryland, and WI = Wisconsin.

[b] A reaction was deemed positive for V6-virulence if sporulation was observed by 21 days after inoculation onto cultivar Nugget.

**Table 2.** Primers used to amplify each diagnostic contig for sequencing

| Primer Pair | Primer Sequence | Contig | Location (*n*)[a] | Amplicon size (bp) |
|---|---|---|---|---|
| 1309F | 5'-GTATCGTCTTACCGATCCATTAAGAATCAGC-3' | 674 | 1408 | 179 |
| 1487R | 5'-TTTGAGAACAGGCCCCGC-3' | … | | --- |
| 91F | 5'-CCGACATCGGTGAAAGGAGTTCATC-3' | 869 | 1611-1612[b] | 74 |
| 164R | 5'-CGCGAAACTGTCAACGAAACATCG-3' | … | | --- |
| 98F | 5'-CGGTGAAAGGAGTTCATCATTTCTTGGA-3' | 869 | | 97 |
| 194R | 5'-GGTCAAGTTAGTCAATGGTGCTGCG-3' | … | | --- |
| 302F | 5'-TTGGCGGTGTGGGTAGAATGC-3' | 1456 | 357 | 118 |
| 421R | 5'-GAATCCGCCTACTTCTTCCTACTTGC-3' | … | | --- |
| 1365F | 5'-CAGGCCTTAGGGCCAGC-3' | 1456 | 1422 | 122 |
| 1486R | 5'-CTCCTGATCGGGTTTCGTACTCG-3' | … | | --- |
| 873F | 5'-CCATCGCACTCTTCTAATGCTGTGC-3' | 2251 | 942 | 130 |
| 1002R | 5'-CAGCGTTGGTGGATGTCACTGTAT-3' | … | | --- |
| 376F | 5'-GAACGTGCCGAAGAATTCTCGCAG-3' | 2407 | 458 | 195 |
| 570R | 5'-TGAAGCCCCGGAGGTATATTTCTTGC-3' | … | | --- |
| 295F | 5'-CAACAAGTTCCTGCTTGGAGTCCTC-3' | 3456 | 352 | 115 |
| 409R | 5'-CGTGCCACTCTACTACAGTCAAGGC-3' | … | | --- |
| 879F | 5'-GCCATCCCTCAGTCAATCGTTTGC-3' | 4382 | 931 | 135 |
| 1013R | 5'-GAAATCGACCGGGAGACTTACTGTGC-3' | … | | --- |
| 556F | 5'-CGGACTAATTCTTGCAGCAAACTTCAACG-3' | 4752 | 626 | 140 |
| 695R | 5'-TAGTTCGATAGTACATGGCCGAGCG-3' | … | | --- |
| 779F | 5'-TTGAGGTGCCTGGACCTTATTACAAGG-3' | 4483 | 791,826,884,886,901 | 124 |
| 902R | 5'-GCAGCCTCGTAAGGTGATGGG-3' | … | | --- |
| 782F | 5'-AGGTGCCTGGACCTTATTACAAGGTTTC-3' | 4483 | | 140 |
| 921R | 5'-GCATTTCTCGTAACGATAAGCAGCC-3' | … | | --- |
| 576F | 5'-CTTGTTTGACTGCACTGGGAGGTC-3' | 5050 | 268 | 118 |
| 693R | 5'-CCGGGATTCAAATTCCGGACGG-3' | … | | --- |
| 221F | 5'-GCCCCTTGATCCTCTATGACATGC-3' | 5050 | 637 | 135 |
| 355R | 5'-GGACTTAGTCCGCCTTGGCC-3' | … | | --- |

[a] Single nucleotide polymorphism (SNP) location based on results from transcriptome sequence data (Gent et al. 2020).

[b] Location of diagnostic insertion/deletion.

**Table 3.** Locked nucleic acid (LNA) probes and flanking primers for differentiating V6 from non-V6 isolates

| Probe or Primer | Sequence |
|---|---|
| **LNA probe**[a] | |
| N2407.WT | 5’-/5HEX/TA AT+C +**A**+AA GG+A AA+TAAG TAG A+AGGC/3IABkFQ/-3’ |
| N2407.P1 | 5’-/56-FAM/TA AT+C +**T**+AA GG+A AA+TAAG TAG A+AGGC/3IABkFQ/-3’ |
| **Flanking primer**[b] | |
| N2407.376F | 5’-GAACGTGCCGAAGAATTCTCGCAG-3’ |
| N2407.570R | 5’-TGAAGCCCCGGAGGTATATTTCTTGC-3’ |

[a] Locked nucleic acid oligonucleotides indicated by (+) with diagnostic single nucleotide polymorphism indicated in bold text.

[b] Flanking primers amplified a 195-bp section of contig 2407.

**Table 4.** Limit of detection of the multiplex locked nucleic acid probe assay and threshold cycle (Cq) values for mixed-race samples run at varying DNA concentrations

| ***P. macularis* template DNA (pg/sample)** | | **FAM fluorophore** | | **HEX fluorophore** | |
|---|---|---|---|---|---|
| **V6** | **Non-V6** | **Mean** | **STD** | **Mean** | **STD** |
| 2000 | None | 31.28 | 0.11 | ND[a] | ND |
| 200 | None | 34.23 | 0.33 | ND | ND |
| 20 | None | 38.65 | 0.49 | ND | ND |
| 2 | None | ND | ND | ND | ND |
| None | 2000 | ND | ND | 29.08 | 0.07 |
| None | 200 | ND | ND | 32.49 | 0.14 |
| None | 20 | ND | ND | 36.32 | 0.55 |
| None | 2 | ND | ND | ND | ND |
| 2000 | 2000 | 29.28 | 3.62 | 28.36 | 0.59 |
| 200 | 200 | 33.06 | 3.36 | 31.65 | 0.54 |
| 20 | 20 | 36.77 | 3.53 | 35.66 | 1.74 |
| 2 | 2 | 38.82 | 3.76 | 38.82 | ND |
| 2000 | 20 | 29.13 | 3.64 | 37.79 | 1.43 |
| 200 | 20 | 32.11 | 2.76 | 35.39 | 1.07 |
| 20 | 2000 | 38.24 | 1.76 | 26.60 | 0.20 |
| 20 | 200 | 35.43 | 0.45 | 30.21 | 0.10 |

[a] The FAM labeled probe (N2407.P1) specifically anneals to the V6 target sequence of fungal isolates collected in Pacific Northwestern region of the US due to one single nucleotide polymorphism located within contig 2407. The HEX labeled probe (N2407.WT) specifically anneals to the non-V6 target sequence containing the alternative allele at the same location. Cq = cycle threshold. ND = not detected.

**Table 5.** Differentiation of *Podosphaera macularis* isolates following a multiplex real-time polymerase chain reaction run with two differentially labeled locked nucleic acid probes

| | | FAM (Cq) | | HEX (Cq) | |
|---|---|---|---|---|---|
| **Race** | **Isolate** | **Mean** | **STD** | **Mean** | **STD** |
| V6 | HPM-198 | ND | ND | 35.08 | 0.86 |
| Non-V6 | HPM-199 | ND | ND | 34.04 | 0.16 |
| Non-V6 | HPM-200 | ND | ND | 27.72 | 0.41 |
| Non-V6 | HPM-201 | ND | ND | 32.01 | 0.08 |
| V6 | HPM-202 | ND | ND | 28.94 | 0.35 |
| Non-V6 | HPM-203 | ND | ND | 34.23 | 0.08 |
| Non-V6 | HPM-204 | ND | ND | 32.95 | 0.08 |
| V6 | HPM-205 | ND | ND | 30.74 | 1.83 |
| V6 | HPM-258 | 35.03 | 0.73 | ND | ND |
| V6 | HPM-368 | 36.03 | 0.29 | ND | ND |
| V6 | HPM-374 | 36.18 | 0.24 | ND | ND |
| V6 | HPM-609 | 34.98 | 0.39 | ND | ND |
| Non-V6 | HPM-663 | ND | ND | 28.29 | 0.06 |
| V6 | HPM-666 | 32.96 | 0.23 | ND | ND |
| Non-V6 | HPM-697 | ND | ND | 30.48 | 0.05 |
| V6 | HPM-704 | 34.78 | 0.34 | ND | ND |
| V6 | HPM-712 | 35.19 | 0.38 | ND | ND |
| V6 | HPM-713 | 36.08 | 0.14 | ND | ND |

| | | | | | |
|---|---|---|---|---|---|
| V6 | HPM-714 | 37.43 | 0.45 | ND | ND |
| V6 | HPM-715 | 34.35 | 0.10 | ND | ND |
| V6 | HPM-716 | 34.44 | 0.20 | ND | ND |
| V6 | HPM-718 | 34.48 | 0.21 | ND | ND |
| V6 | HPM-719 | 35.46 | 0.24 | ND | ND |
| V6 | HPM-724 | 34.44 | 0.30 | ND | ND |
| V6 | HPM-726 | 34.62 | 0.14 | ND | ND |
| Non-V6 | HPM-956 | ND | ND | 33.73 | 0.25 |
| Non-V6 | HPM-967 | ND | ND | 31.51 | 0.48 |
| Non-V6 | HPM-1040 | ND | ND | 33.42 | 0.09 |
| Non-V6 | HPM-1053 | ND | ND | 28.42 | 0.05 |
| Non-V6 | HPM-1055 | ND | ND | 28.66 | 0.06 |
| Non-V6 | HPM-1056 | ND | ND | 31.30 | 0.16 |
| Non-V6 | HPM-1059 | ND | ND | 34.55 | 0.15 |
| Non-V6 | HPM-1064 | ND | ND | 34.07 | 0.06 |
| Non-V6 | HPM-1066 | ND | ND | 33.75 | 0.32 |
| V6 | HPM-1068 | 35.33 | 0.18 | ND | ND |
| V6 | HPM-1075 | 31.70 | 0.07 | ND | ND |
| V6 | HPM-1077 | 31.37 | 0.17 | ND | ND |
| Non-V6 | HPM-1078 | ND | ND | 32.78 | 0.10 |
| Non-V6 | HPM-1081 | ND | ND | 30.58 | 0.06 |
| Non-V6 | HPM-1084 | ND | ND | 33.65 | 0.29 |

| | | | | | |
|---|---|---|---|---|---|
| Non-V6 | HPM-1099 | ND | ND | 35.11 | 0.28 |
| Non-V6 | HPM-1101 | ND | ND | 33.29 | 0.14 |
| Non-V6 | HPM-1107 | ND | ND | 33.82 | 0.29 |
| V6 | HPM-1112 | 28.64 | 0.18 | ND | ND |
| V6 | HPM-1120 | 32.49 | 0.13 | ND | ND |
| V6 | HPM-1122 | 34.84 | 0.12 | ND | ND |
| V6 | HPM-1123 | 35.95 | 0.58 | ND | ND |
| Non-V6 | HPM-1124 | ND | ND | 36.11 | 0.13 |
| Non-V6 | HPM-1126 | ND | ND | 31.54 | 0.13 |
| Non-V6 | HPM-1137 | ND | ND | 35.05 | 0.21 |
| Non-V6 | HPM-1151 | ND | ND | 36.83 | 0.41 |
| Non-V6 | HPM-1152 | ND | ND | 32.96 | 0.14 |
| Non-V6 | HPM-1182 | ND | ND | 34.32 | 0.42 |
| Non-V6 | HPM-1183 | ND | ND | 34.67 | 1.98 |
| Non-V6 | HPM-1190 | ND | ND | 31.74 | 0.37 |
| Non-V6 | HPM-1193 | ND | ND | 28.81 | 0.08 |
| Non-V6 | HPM-1196 | ND | ND | 31.10 | 0.10 |
| Non-V6 | HPM-1198 | ND | ND | 35.66 | 0.12 |
| Non-V6 | HPM-1200 | ND | ND | 29.31 | 0.18 |
| Non-V6 | HPM-1203 | ND | ND | 27.66 | 0.08 |
| V6 | HPM-1216 | 29.91 | 0.70 | ND | ND |
| V6 | HPM-1217 | 29.09 | 0.43 | ND | ND |

| | | | | | |
|---|---|---|---|---|---|
| V6 | HPM-1218 | 28.75 | 0.15 | ND | ND |
| V6 | HPM-1220 | 29.52 | 0.01 | ND | ND |
| V6 | HPM-1221 | 31.08 | 0.21 | ND | ND |
| V6 | HPM-1222 | 30.32 | 0.19 | ND | ND |
| Non-V6 | HPM-1223 | ND | ND | 36.36 | 0.47 |
| Non-V6 | HPM-1224 | ND | ND | 30.62 | 0.08 |
| V6 | HPM-1226 | 33.18 | 0.11 | ND | ND |
| V6 | HPM-1227 | 34.35 | 0.18 | ND | ND |
| V6 | HPM-1229 | 33.47 | 0.15 | ND | ND |
| V6 | HPM-1230 | 37.01 | 2.26 | ND | ND |
| V6 | HPM-1231 | 28.10 | 0.51 | ND | ND |
| V6 | HPM-1232 | 29.25 | 0.13 | ND | ND |
| Non-V6 | HPM-1233 | ND | ND | 29.27 | 0.07 |
| Non-V6 | HPM-1234 | ND | ND | 28.82 | 0.41 |
| Non-V6 | HPM-1235 | ND | ND | 30.00 | 0.30 |
| Non-V6 | HPM-1236 | ND | ND | 33.72 | 0.29 |
| Non-V6 | HPM-1237 | ND | ND | 30.70 | 0.07 |
| Non-V6 | HPM-1238 | ND | ND | 29.26 | 0.11 |
| Non-V6 | HPM-1239 | ND | ND | 30.11 | 0.12 |
| Non-V6 | HPM-1240 | ND | ND | 32.35 | 0.11 |
| Non-V6 | HPM-1241 | ND | ND | 34.25 | 0.10 |
| V6 | HPM-1242 | 32.40 | 0.18 | ND | ND |

| | | | | | |
|---|---|---|---|---|---|
| V6 | HPM-1243 | 32.19 | 0.06 | ND | ND |
| V6 | HPM-1244 | 31.62 | 1.28 | ND | ND |
| V6 | HPM-1251 | 33.13 | 0.18 | ND | ND |
| Non-V6 | HPM-1252 | ND | ND | 33.04 | 0.37 |
| Non-V6 | HPM-1253 | ND | ND | 35.67 | 0.08 |
| Non-V6 | HPM-1254 | ND | ND | 33.01 | 0.13 |
| Non-V6 | HPM-1256 | ND | ND | 30.31 | 0.13 |
| Non-V6 | HPM-1257 | ND | ND | 31.01 | 0.05 |
| Non-V6 | HPM-1258 | ND | ND | 32.51 | 0.07 |
| Non-V6 | HPM-1260 | ND | ND | 29.29 | 0.07 |
| Non-V6 | HPM-1261 | ND | ND | 29.52 | 0.08 |
| V6 | HPM-1263 | 33.02 | 0.19 | ND | ND |
| V6 | HPM-1264 | 32.59 | 0.36 | ND | ND |
| V6 | HPM-1265 | 34.25 | 0.19 | ND | ND |
| V6 | HPM-1266 | 34.58 | 0.20 | ND | ND |
| V6 | HPM-1267 | 35.30 | 0.17 | ND | ND |

[a]The FAM labeled probe (N2407.P1) specifically anneals to the V6 target sequence from fungal isolates collected in Pacific Northwestern region of the U.S. due to one single nucleotide polymorphism located within contig 2407. The HEX labeled probe (N2407.WT) specifically anneals to the Pacific Northwestern non-V6 target sequence containing the alternative allele at the same location. Cq = cycle threshold. ND = not detected.

**Table 6.** Multiplex assay with powdery mildew fungi collected from other host species

| Host[a] | Host common name | FAM (Cq)[b] | | HEX (Cq)[b] | |
|---|---|---|---|---|---|
| | | Mean | STD | Mean | STD |
| *Convolvulus arvensis* | Field bindweed | 39.1 | 0.5 | 37.9 | 0.8 |
| *Plantago major* | Broadleaf plantain | 35.6 | 0.6 | 35.8 | 0.6 |
| *Eucalyptus* sp. | Eucalyptus | ND | ND | ND | ND |
| *Fragaria* sp. | Strawberry | 38.7 | ND | 38 | 1.7 |
| *Lathyrus odoratus* | Wild sweet pea | ND | ND | ND | ND |
| *Malus domestica* | Apple | ND | ND | 36.7 | 0.3 |
| *Prunus avium* | Sweet cherry | ND | ND | 39.1 | ND |
| *Rosa* sp. | Rose | ND | ND | 37.7 | 0.3 |
| *Syringa vulgaris* | Lilac | ND | ND | 39.7 | ND |
| *Taraxacum* sp. | Dandelion | 38.5 | ND | ND | ND |
| *Trifolium* sp. | White clover | 37.4 | ND | 38 | 1.3 |
| *Vitis vinifera* | Wine grape | 35.9 | 0.9 | 39 | 1.1 |
| *Zinnia* sp. | Zinnia | 32.4 | 0.1 | 32.7 | 0.2 |

[a] Powdery mildew isolates were collected from plant host species in the Pacific Northwest and ITS-sequence verified for confirmation of genus of the fungus.

[b] The FAM-labeled probe (N2407.P1) specifically anneals to the V6 target sequence of hop powdery mildew fungal isolates collected from the Pacific Northwestern region of the U.S. due to one single nucleotide polymorphism located within contig 2407. The HEX-labeled probe (N2407.WT) specifically anneals to the non-V6 target

sequence of Pacific Northwestern hop powdery mildew fungal isolates containing the alternative allele at the same location. Cq = cycle threshold. ND = not detected.

**NODE_2407**

TCTATGATCTGATTATTTTCCTGACAAGCACGATTATATCCAACATCCTG
CTTCATCAACTGAAATGGAATAAGTAGTGAAATAATGATAAAGCTTGTGG
AACTGAAAAAAACTTGATCGCAGTGTCTTTTCGGTATGAATTTATATTCCA
TGTACCAAGAGCTTAACCCTTTTCTTTTTTGTCATGCTTTATTTTTACAA
ATCAGCGACTTTAGGAATCAAGGCCTTGAACGTAGGGCATATGCACAGAT
AATCCTGAGCGGATTCTTAGACATGTGTATCGTAGGTTAAGTTTGTGATC
CACGAAGCCTAAACTTATCGTTGTGCAGAATGGCTGTGATAGGATTATAT
TGAACTATATCTTTCATCATAGATTGAACGTGCCGAAGAATTCTCGCAGA
GTTGTATCTTTTATCTCCTATTAACCCTACTAGAGGGCTATCTGGGGGTG
AATAATCAAAGGAAATAAGTAGAAGGCTTTCTCTCAGAAGGGCCACTGAT
TCAATTAAGTTTTACGGTGTAGCAAAACAATATCCAGCATTTTTGCAAGA
AATATACCTCCGGGGCTTCAAATCTCTTTACAACATTGAGGAGTGAGGAT
ACGATTCTGGTACACGAGGAAGTGTGGTTGTGGCTAGGTATTGTAAAAAA
TGGCTGAAAGAGGTGTTGCAGCAAAATATCCTTTTTGTATTCTTCTTAGC
GTCGGGCACCTCAGATTAATAGAAAAATTTGAAGCTCTAGTCCTCCGTTT
CCCCTGAAGTTGATCTTTAGTCATTAATACACTTGAACGCCAATATCACA
GTCCGTACTCGCTTTCCTTATACACGCCTTAGTCAGTATCGCTCGGCCTT
CTCATGTAAGTATCAATTGCTGCGCTGAAGGCCGCAAATCCAGCACAGCC
GACTGCGGCTGCTTGTGGACCAGCAGAAGCAGCCAAAACACCACCGGTGA
TACATCCCGCCAAAATACCATTTGTTAGGTCATTCTTAGCTCGATAGCCT
TCGATGCAGCACTCGGTACCAGCAAATATAGCGCCAACTTTACCGAAATT
TTTGGCTGAGGAAAAAGAGCGTGTGCCCATATCTTTAAAGCCTGCTTTGA
GCTGTTCTCGAAGAGGTTTTGACGGGGCGGGCGAATTGTGGAGAGGCGTA
TCGTATTGCATTGAAGCCATAAAAAGCCCAAAGACGCCACCTAGTCCAAA
CCCCATTACCCCTGATATAACAGTTTTTGCGGGACAGGATTCCATGATGA
TTTGCATACTTTTGATTGCCAACTTAGTTTGTTCATCAATCTCATTTGTC
CCGCCAGCTCCAGCCCCAGGACTGGCAAACAGCGGACCGTTCATCCTAAC
GCGATTATTAGAGTTGCCTCAATTACCCTCTGTACGGGATTTTTGTCTCT
GGGAATCGCTGATACCAGACTGCTGGGTGCCAGATATACGCTGATACGAG
ACGCAAACTGCTATCTTGTACTGACCATATACTT

> F_primer
GAACGTGCCGAAGAATTCTCGCAG

> R_primer
TGAAGCCCCGGAGGTATATTTCTTGC

> Probe
FAM- TAATCTAAGGAAATAAGTAGAAGGC-BHQ

**The SNP in the target sequence is highlighted in pink. The "T" polymorphism with an LNA probe. The alternate sequence contains an "A".**

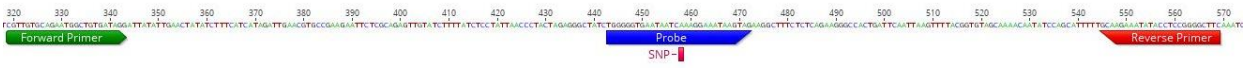


**Figure 1.** Above: Sequence of Contig 2407 with locations of primers and probes highlighted. Below: Primer and probe design in Geneious (R11.1) with diagnostic SNP highlighted in pink.

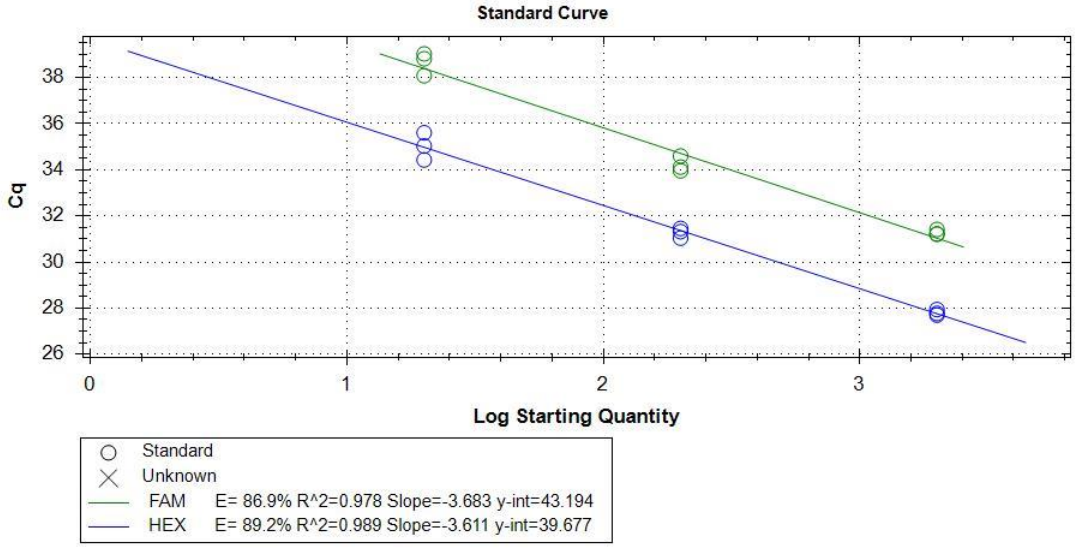


**Figure 2.** Standard curves generated (Bio-Rad CFX Manager 3.1) using 10-fold serial dilutions of *Podosphaera macularis* DNA. Concentrations ranged from 2000 pg to 20 pg of DNA per reaction. Curves represent three biological replicates each of *P. macularis* isolates HPM-666 (V6) and HPM-1040 (non-V6).

# GENERAL CONCLUSIONS

Hop powdery mildew, caused by the pathogen *Podosphaera macularis,* is one of the most important diseases on hop worldwide. Widespread deployment of hop cultivars with R6-based resistance in the Pacific Northwest has led to a population of *P. macularis* that has overcome said resistance. These isolates can persist on hop cultivars that were susceptible prior to 2012, in addition to those cultivars that possess R6-based resistance. This means that powdery mildew found on previously susceptible hop cultivars must undergo screening on a differential set of cultivars in order to determine which race is present. The objective of this project was to develop a PCR-based assay to detect V6 isolates of *Podosphaera macularis* derived from the Pacific Northwest. The resulting multiplexed real-time PCR assay was validated against 46 V6 and 54 non-V6 *P. macularis* isolates collected from the United States and Europe. The assay had perfect discrimination of V6-virulence among isolates of *P. macularis* originating from the western U.S. but failed to predict V6-virulence in three isolates collected from Europe. Contig 2407 used for assay development contains a SNP that is associated with V6-virulence in the Pacific Northwest, but this SNP is not necessarily a causal mutation that confers virulence to the pathogen. As this assay was designed from isolates collected solely from the Pacific Northwest, it is not unexpected that V6 isolates originating from other countries were not detected. The assay has practical applications in hop breeding, epidemiological studies, and other settings where rapid confirmation of pathogen race is needed.

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# APPENDIX

**Protocol for maintenance of *Podosphaera macularis* isolates**

*Materials*

Biocontainment hood

Petri dishes (100mm x 15mm)

Made double Petri dishes

Water

70% ethanol in spray bottle

95% ethanol

Rooting solution

Susceptible plant (cv. Symphony)

Scissors

Dissecting microscope

Transfer tools

10% bleach water

*Procedure*

1. Select susceptible plants (cv. Symphony) grown in a powdery-mildew-free greenhouse that have young, unfurled leaves at the first node. Avoid leaves that are chlorotic or have damage (such as thrips damage). Surface of the leaves are misted with 70% ethanol and rinsed with water after 30 to 60 seconds for approximately 30 seconds to ensure potential sulfur and ethanol residues are removed. Allow plants to dry.

2. After plants are dry, detach the first node of the plant by cutting the stem approximately 1-2 inches below the leaves, also remove stem above the leaves then place in a double Petri plate with water in bottom.

3. Infected tissue (generally leaves or cones) is brought in from the field. Infected areas of tissue are dabbed onto powdery mildew free leaves prepared above. Generally 2 leaves in individual double Petri plates are used per isolate. Newly inoculated cultures are placed in a growth chamber for 2 weeks to establish and sporulate. Alternatively, an isolate may already be growing and can be transferred as below.

4. Isolates should be transferred to new leaves every 2 to 3 weeks to ensure the health of the isolate. Isolates should be transferred in the biocontainment hood to decrease the probability of isolates becoming contaminated and to prevent escape of isolates from outside of Oregon as per APHIS permit requirements.

5. Under a dissecting scope transfer small quantities (1 to 10 chains) of *P. macularis* using either a small insect needle or eyelash transfer tool to a prepared leaf. Repeat 7 to 10 times per leaf. Transfer tools should be sterilized in 95% ethanol for at least 60 seconds between isolates. In addition, hands and surfaces of hood should be sprayed with 70% ethanol between isolates.

6. Petri plate containing the newly inoculated leaf should be labeled and placed in growth chamber.

7. Leaf containing the original culture can either be resealed and kept as a backup culture or discarded. If discarding the, leaf should be removed from the double Petri plate and sealed in a plastic bag or in single Petri plate. The double Petri plate and water should be placed in 10% bleach water before being washed. The discarded leaves should be autoclaved before being thrown away.

**Protocol for obtaining a clonal (single-chained) isolate of *Podosphaera macularis***

*Materials*

Infected plant material with sporulating colonies

Biocontainment hood

Dissecting microscope

Transfer tools

70% ethanol in spray bottle

95% ethanol

10% bleach water

Healthy leaves prepared in detach system (see maintenance of *P. macularis* isolates)

*Procedure*

1. Obtain an infected hop leaf with sporulating colonies of *P. macularis.* Pick a single colony that is healthy (no or few contaminates present). Sterilize biocontainment hood by spraying surface with 70% ethanol. Also sterilize transfer tools by soaking in 95% ethanol for at least 60 seconds.

2. In the biocontainment hood with a dissecting microscope use the transfer tool to select a single conidiophore from the previously selected colony. Conidiophores are easiest to isolate around the edge of the colonies. Move the conidiophore to the healthy leaf. Repeat 7 to 10 times. Transfer tools should be sterilized in 95% ethanol

for at least 60 seconds between isolates. In addition, hands and surfaces of hood should be sprayed with 70% ethanol between isolates.

3. Petri dish containing the newly inoculated leaf should be labeled and sealed with parafilm and placed in growth chamber. The already infected hop leaf can either be resealed and placed back in the growth chamber as backup culture or can be discarded. If discarded the leaf should be removed from the double Petri dish and sealed in a plastic bag or in single Petri dish. The double Petri dish and water should be placed in 10% bleach water before being washed. The discarded leaves should be autoclaved before being thrown away.

4. In 10 to 14 days, transfer conidia from only one of the colonies formed from the above inoculations to healthy hop leaves. These leaves should be labeled and placed in growth chamber. The resulting infection will be a clonal isolate of *P. macularis* and can be maintained as described in “Protocol for maintenance of *Podosphaera macularis* isolates”.

**Protocol for Chelex extraction of *Podosphaera macularis***

Adapted from:
Brewer, M. T., and Milgroom, M. G., 2010. Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator*, from diverse *Vitis* species. BMC Evolutionary Biology 10:268.

*Materials*

Biocontainment hood

Clear office tape

70% ethanol

Forceps

Sterile, 1.5mL microcentifuge tube

5% chelex (Sigma C7901-25G) in a sterile 50 mL Falcon-type tube

Sterile silica beads (400 micron)

P200 pipette and P200 filter tips

Vortex

Microcentrifuge

Block heater at 95°C

Negative 80 freezer

*Procedure*

1. Extraction works best with colonies 1 to 3 weeks old and free of contamination.

2. Working with gloves in a sterile biocontainment hood, remove any exposed sticky part of office tape and wipe down tape dispenser with 70% ethanol. Using forceps pull about 2.5cm piece of tape and remove from dispenser.

3. Touch the tape to the mildew colony repeatedly until the tape is covered in conidia and no longer sticky.

4. Put the conidia-covered tape into a microcentrifuge tube and close the tube. Label tube with appropriate information.

5. Hood and gloves should be wiped down with 70% ethanol between each isolate being harvested for conidia. The above steps should be repeated with all isolates to be harvested for conidia.

6. Place 100ul of 5% Chelex solution in each tube. Invert the Chelex suspension several times to re-suspend Chelex beads and quickly remove 100µl with a P1000 Invert Chelex between each tube to keep beads suspended.

7. Vortex tubes about 15 seconds, push tape down with sterile pipette tip if needed, vortex again for 15 seconds

9. Place tubes in a heat block set to 95°C for 15 minutes. Vortex tubes for 20 seconds and return them to the heat block for additional 15 minutes. Remove tubes from the heat block and spin in centrifuge for approximately 5 seconds (quick spin).

10. Let tubes cool at room temperature for approximately 30 minutes. This makes it easier to remove the supernatant but can be skipped if needed.

11. Remove supernatant from the tape and Chelex beads with an uncut filter tip (usually about 50 to 75µl of liquid) and transfer to a new, sterile microcentrifuge tube. Label tube appropriately and store in -20°C. Use 1µl of solution in PCR reactions.

**Protocol for softwood cuttings of hop**

*Materials*

Plants of desired hop cultivar

Scissors

Rooting hormone (see “rooting hormone solution” protocol)

Oasis tray wedge media

Solid-bottom tray

Water

*Procedure*

1. Place Oasis tray in a solid-bottom tray and fill about ½ full of water making sure the Oasis wedges are saturated.

2. Select plant material and remove bine from plant

3. Cut a single node (including leaves) from the removed bine. Remove one of the leaves and dip the stem end of the cutting in rooting hormone (do not get rooting hormone on the leaf). Insert cutting in Oasis wedge.

> Note: in hot weather it may be necessary to remove part of the remaining leaf to reduce transpiration and wilting of the cutting.

4. Label the tray with cultivar and date and make certain the tray is about ½ full of water on a regular basis.

5. Cuttings should be rooted and ready for up potting in about two to three weeks. When they are rooted, pot into desired size pot containing a slow release fertilizer. It is very important to cover the wedge with soil to prevent drying out, but it is also important to make sure the buds don’t get covered up or else the cutting won’t take.

**Protocol for rooting hormone solution**

*Materials*

2x 500mL glass bottles

nitrile gloves

indole butyric acid

boric acid (granular)

scale

weigh boats

500 mL graduated cylinder

95% ethanol

deionized water

50mL beaker

*Procedure*

Before making these solutions, put on nitrile gloves.

1. Indole butyric acid:

    a. add 1 gram of indole butyric acid to 500 mLs of 95% ethanol in a glass bottle. Mix until dissolved.

2. Boric acid:

    a. add 1 gram of granular boric acid to 500mLs of deionized water in a glass bottle. Mix until dissolved.

Store both of these solutions at 4°C

The same day softwood cuttings are being made:

1. Mix the solutions above at a 1:1 ratio for rooting hormone solution. In general, 10 mLs of each solution is mixed together in a small 50mL beaker.