Conferring Downy Mildew (*Pseudoperonspora humuli)* Resistance in Hop (*Humulus lupulus*)

by

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US Hop Production

Historical Overview

Hops are thought to be native to Asia. The three known hop species are *Humulus lupulus*, *H. japonicus*, and *H. yunnanensis*. *H. lupulus* is the only widely cultivated crop species of the three, although *H. japonicus* can be used ornamentally. Several subspecies exist within *H. lupulus*, which include a European, Japanese, common hop subspecies *var. lupulus*, and three native North American subspecies. The North American subspecies include *var. neomexicanus*, *var. pubescens*, and *var. lupuloides*; these represent Western North America, Midwestern USA, and Central and eastern North America (Small, 1980). Many of today's most popular cultivars are bred using the European lineage. In recent decades, however, North American cultivars have been incorporated into hop breeding to explore native diseases resistance and new flavors (Lemmens, 1997). The breeding of hops in a scientific manner can be dated back to the start of the hop breeding program at Wye College in 1904 (Neve, 1986).

Modern Overview

Hop *(H. lupulus)* is a perennial dioecious plant that is grown for the strobili or cones that grow on the female plants only. These cones are used almost exclusively for bittering and aroma in beer brewing. A small market for essential oils and medicinal products derived from hops exists as well.

In commercial hop production, bines are grown on a trellis system, usually 5-8 meters tall, at a population density of 2200 plants (hills) per hectare. The bines are cut and cones are stripped mechanically from the plants, and then dried on large bed dryers with cones layered at a depth of around 90 cm (Sirrine et. al., 2010). They can then be compressed into 92 kg bales of whole

cone hops, to be processed by a wholesaler. Further processing turns whole cone hops into pellets. To process hops into pellets, it is first milled into powder, before pressed into pellets by a pellet mill. Pelleted hops are a more consistent product for brewers to use. Hops pellets have greater uniformity of moisture, oil content, vegetative matter, and density than whole cone hops. The hops pellets packaging is flushed with inert gas such as nitrogen to displace oxygen. Oxygen rapidly oxidizes hops and leads to off-flavors and undesirable aromas. Hop pellets in proper packaging have a greater shelf-life than whole leaf hops, three years for pellets compared to a year for dried whole cone hops (Yakima Chief – Hopunion, 2015). A small market exists for fresh or wet hops, which are used for brewing within 24 hours of harvest. Wet hops represent less than 1% of the market due to perishability and because they tend to be used solely by small scale brewers. Wet hops are harvested at about 80% moisture and generally used towards the end of the brew process to impart fresh hop flavors and aromas. These tend to be volatile flavors which start to fade quickly following harvest (Moorhead, 2017). Around 45% of hops are converted to hop extract, and 40% are pelleted (Hughes, 2003). According to the 2017 USDA Hop Report (National Agricultural Statistics Service, 2017), 75% of the United States hop crop was produced in the state of Washington. Another 24% in Idaho and Oregon, and the remainder in small yards throughout the country. Hops grown commercially are generally propagated clonally, from rootstock or rhizomes.

The average yield for ‘Cascade’, the most widely grown cultivar was 2,195 kg ha^{-1} in 2017, and with the 2017 average price per kilogram of hops at $13.05, the revenue was around $70,804 ha^{-1} for Cascade. Hopyard costs are easily $67,164 ha^{-1}, including trellis maintenance, labor, and harvest costs (Sirrine et al, 2014). With yield reductions reported anywhere from 0

to 100% combined with quality reductions can quickly bring demand for a crop to zero (Johnson et al., 2009).

Propagation Method

Hops are propagated from rhizomes. Rhizomes can be acquired from certified sources such as the Nation Clean Plant Network-Hops (University of California, 2018). A grower has the choice to either purchase liners (starter plants) or rhizomes. Liners are greenhouse trays or flats of seedlings. These can be transplanted into a commercial production field. According to Cochran (2016), rhizomes are often cheaper than liners, however they have a longer establishment time. Rhizomes are planted as soon as the soil thaws, while liners can be planted anytime between April and June, depending on the weather in the growing region.

Management Practices

Hop plants take three to four seasons before they reach full maturity and maximum yield. Due to this, fertility management is of upmost importance to track during season one compared to subsequent seasons. First year hop plants require about a third to half of the amount of nitrogen required during full production years. Throughout the first growing season nitrogen should be applied at 78.5-84 kg ha^{-1} and 112-168 kg ha^{-1} the following years. Furthermore, nitrogen application should be done as a split application; half is applied in the spring at first growth and the second half is applied approximately five weeks later (Cochran, 2016).

Phosphorus and potassium levels are also important to track and apply throughout the growing season. Recommended fertilizer rates are 22.4-33.6 kg ha^{-1} of phosphorous and 90-168 kg ha^{-1} of potassium, which can be applied in conjunction with the first nitrogen application.

During the root establishment process, the soil should be kept moist, approximately 2.54 cm of water per week. One may rely on rainfall, but supplement with irrigation as needed. Once root establishment is complete, plants require 3.8 cm of irrigation per week (Cochran, 2016). Hop yards should have irrigation equipment installed to ensure adequate moisture is available. Drip irrigation is preferred to overhead sprinklers, because it applies water directly to the roots which reduces evaporation. Another advantage of drip irrigation is that it keeps water off foliage and reduces the likelihood of waterlogged soil, which decreases pressure from diseases such as downy mildew (*Pseudoperonspora humuli),* powdery mildew *(Podosphaera macularis),* and Verticillium wilt (*Verticillium albo-atrum* or *Verticillium dahlia*) (Sirrine et al., 2014).

The impact of downy mildew on hop production may be reduced by several methods. One is to select resistant cultivars. 'Wye Northdown', 'Wye Challenger', 'Newport', and 'Perle' are cultivars that were bred for strong resistance to DM. Another method is application of fungicides. Some of the most effective fungicides are cyazofamid, ametoctradin + dimethromorph, mandipropamid, cymoxanil, famoxadone, and fosetyl-Al (Brown-Rytlewski et al., 2016). Effective fungicide programs spray approximately every 10 days. Products like copper and oils are used to mitigate diseases, but are not as effective as the aforementioned fungicides. Drawbacks are pre-harvest intervals and limits on the maximum amount of fungicide that can be applied per year. A pre-harvest interval is the minimum amount of time that must pass between the last pesticide application and when the hop cones are harvested. The cost of these intensive fungicide programs is often around \$1,483 ha^{-1} per year (Sirrine et al., 2014). Spring pruning or crowning is quite effective as well. Pruning means cutting back the shoots above the ground, while crowning refers to the removal of the top part of the hop

crown just prior to or following shoot emergence (Darby and Calderwood, 2016). This removal of the first flush of growth in the spring can be done mechanically or chemically with an herbicide. The first flush of growth often can include infected shoots, so removing them limits the amount of disease presence. Since downy mildew infections can be systemic, it is important to start with pathogen-free rhizomes and control infection. Once DM becomes established in a hopyard, it is difficult to eliminate it. This is one reason why resistance is so valuable. Hopyard sanitation is important to reduce inoculum present for future infection. Uncut bines and leftover plant material should be removed (Infante-Casella and Bamka, 2017).

Harvest and Storage

Harvesting of hops in Michigan occurs in late August and the end of September. Sirrine et al., (2014) stated that several factors affect the timing of hop harvest including weather, cone moisture content, and pest and disease issues. Cone moisture should be between 72 and 78% moisture at the time of harvest for optimal quality. Timing of harvest is crucial for growers since prime harvest conditions for hop harvest only last 7-10 days once hops are ready for harvest. Signs of harvest readiness include the cone texture, feeling papery and dry, the lupulin inside the cone turning dark yellow, and cones that stay compressed when crushed between the fingers. Crushed cones will leave behind a sticky residue and offer a strong aroma (Sirrine et al., 2014). Commercial growers use the cutting method, where the hop bines are cut from the hop trellis, just above the base of the plant and fed into a cone picking machine. This machine strips the bines, and separates cones from leaf and stem material using air and sets of baffles and sieves. This cone picking machine may be mobile, traveling through the field, or a stationary unit requiring bines to be transported out of the field to the cone picking machine (Sirrine et al., 2010). Non-commercial growers use what is known as the picking method. This

method includes picking the hops from the bines, while the bine remains attached. According to Roberts (2016), the picking method carries several benefits, once of which being that you can pick the cones that are ready to be harvested, while leaving the rest to harvest once they are fully mature, thus lengthening the harvest window and increasing overall yield. Furthermore, picking allows the grower to leave the bine in place until later in the fall, allowing the leaves to fall off. By leaving the bine up post-harvest, nutrients and energy can return to the roots to prepare for dormancy. With the cutting method of harvest, more stress is induced on the plant (Roberts, 2016). However, in commercial hopyards the cutting method is preferred to the picking method due to labor constraints and harvest timing. Large scale operations have a lot of hops to pick in a short time, which is easier to do via mechanical picking. The Wye Hops Ltd. breeding program of the United Kingdom has developed a number popular dwarf cultivars. Dwarf cultivars are grown on 2.3 to 3 meter trellises in continuous hedges. This allows for less costly hopyard installation, with reduced inputs of labor and mechanical harvest requirements. Cultivars adapted to conventional trellis systems (5 to 8 meters tall) do not yield well when grown on low trellises (Darby, 2005).

In addition to these field tests, growers do have the option of sending in cone samples for testing to ensure proper harvesting conditions. Laboratories test for dry-matter content of the hop cones. Dry-matter, which is the opposite of percent moisture by weight serves as an indication of how heavy the cones will be once dried. Dry-matter information is combined with the standard harvest region for a growing area for the cultivar being grown to determine if the specific hop crop is ready to be harvested.

Once cones are removed from bines, they can either be dried or used immediately. Cones that are used immediately after harvest are used to brew wet-hopped seasonal ales, while those that

are dried, are vacuum-sealed and stored in refrigeration to be used at a later time. Cone drying methods are dependent upon the quantity of the hops harvested, the facilities available and the budget that a company or individual has. Hops that are dried commercially are placed in a commercial dryer that is located in a sealed room with a dehumidifier, while hops that are dried in non-commercial settings are often dried on a screen in the sun or in another warm location. Regardless of drying method, hops dried at a temperature below 60 °C will generally result in a high-quality product. Hops should be dried to between 8 to 12% moisture to keep them from molding or otherwise spoiling (Sirrine, 2014). If hops bale moisture is greater than 12%, there is an increased chance of composting and spontaneous combustion of bales (Gorst Valley Hops LLC, 2013).

Once harvest is complete, bines can be either burned or composted. Bines should be cut being careful not to disturb the rhizome. If the grower is in an area that experiences winter conditions where temperature fall in the single digits, Roberts (2016), suggests adding a moderate blanket of mulch or organic debris on top of the hill where the rhizome is planted to both feed the soil as well as insulate the rhizome from the coldest temperatures. However, caution should be taken not to insulate the ground too much as the rhizome benefits from the ground experiencing a hard freeze to help it prepare for the coming spring (Roberts, 2016). If composted, bines, leaf and stem materials should be kept away from the soil near the hop plants to reduce the chance of spreading disease (Sirrine et al., 2014).

U.S. Production Information

In 2017, Cascade made up over 13% of the U.S. harvest by weight. Cascade has been the number one hop cultivar for at least the last 10 years. Figure 1 shows the top ten hop cultivars

grown in the U.S. based on production from 2007 to 2014. The top two cultivars remain the same, but popularity of the third ranked cultivar and below changes frequently.

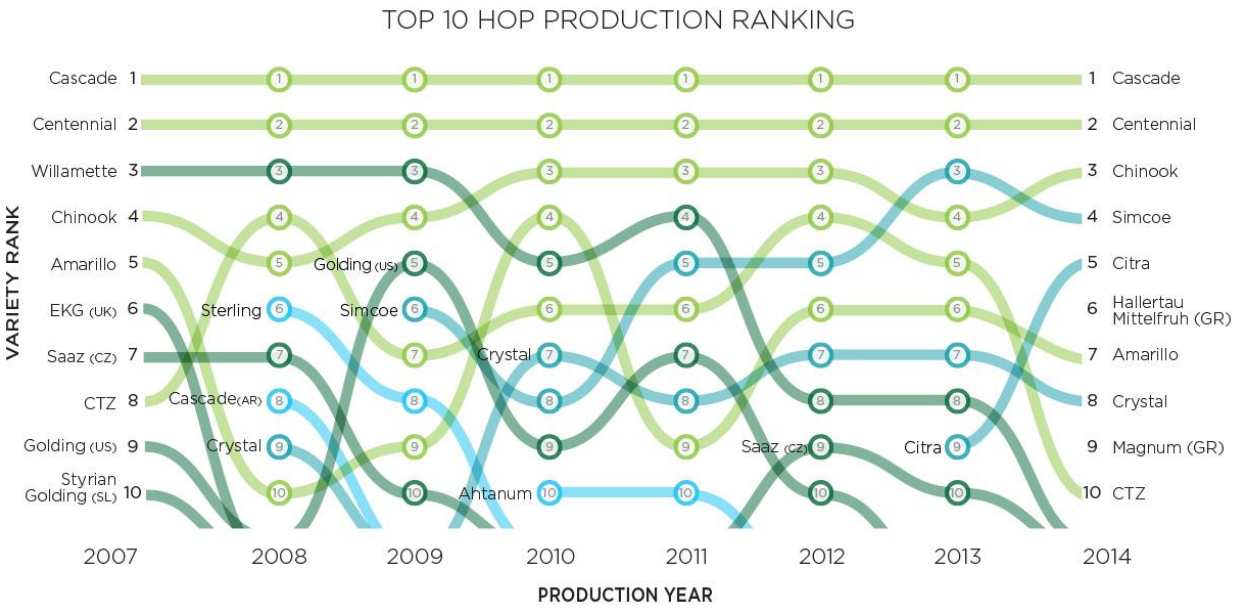

Figure 1: Hop Production Ranking (Brewers Association,2016)

The US market for hops is still expanding. Production went from just under 35,763,000 kg in 2015 to over 47,340,000 kg in 2017. Growing area also increased from 17,658 ha to 21,563 ha from 2015 to 2017. Simultaneously, the average price per kg increased from \$9.66 kg^{-1} to \$13.05 kg^{-1} (National Agricultural Statistics Service, 2017). The increase in production and increase in price is indicative of a growing market. There are approximately 6,200 breweries operating in the U.S., with 2,500 more in the planning stages (Scott, 2018). This also describes a growing market; thus, an opportunity exists for hop breeders to develop and market new hop cultivars with unique flavor and aroma characteristics, which are resistant to a larger number of the most problematic diseases.

Hop Biology

Ploidy and Genome

Hop plants have nine pairs of autosomal chromosomes, and a pair of sex chromosomes, X and Y (McAdam et al., 2013). Hop tolerates multiple ploidy levels, including tetraploid and triploid types. There are examples of popular commercial cultivars, with Crystal', 'Galaxy', and 'Williamette' being triploid. Triploid hop are reported to be more vigorous when compared to diploid and tetraploid cultivars (Haunold, 1980). Tetraploid hop plants can occur spontaneously from seed, or be induced with colchicine. Crossing a diploid parent to a tetraploid parent results in triploid offspring. The hop genome is estimated to be between 2.57 and 2.8 Gb (Matthews et al., 2013; Natsume et al., 2015) which is quite large compared to its closes relative, *Cannabis sativa*, and is similar in size to that of the human genome (3 Gb). This may be due to genome-wide duplication or extensive repetitive element content (Matthews et al., 2013).

Downy Mildew

Life Cycle

Pseudoperonspora humuli, the causal agent for Downy Mildew (DM), is a fungus-like pathogen of the Oomycete class that causes local and systemic infections in hops (Henning et al., 2015). DM causes significant reductions in yield and hop quality, depending on variety and disease severity (Linzotte, 2015). DM oospores can overwinter in hop buds and crowns, leading to infected shoot emergence in the spring. Infected shoots produce sporangia, which release zoospores. Sporangia can move on air currents. Zoospores move around in water on the leaf tissue and enter leaves through open stomata. These Zoospores are responsible for most of the infection and blighting that occurs on leaves, cones, and shoots (O'Neal et al., 2015).

Figure 2 below shows the DM life cycle, specifically how zoospores continue to infect new tissue throughout the growing season. This happens from water splashing due to rain or wind.

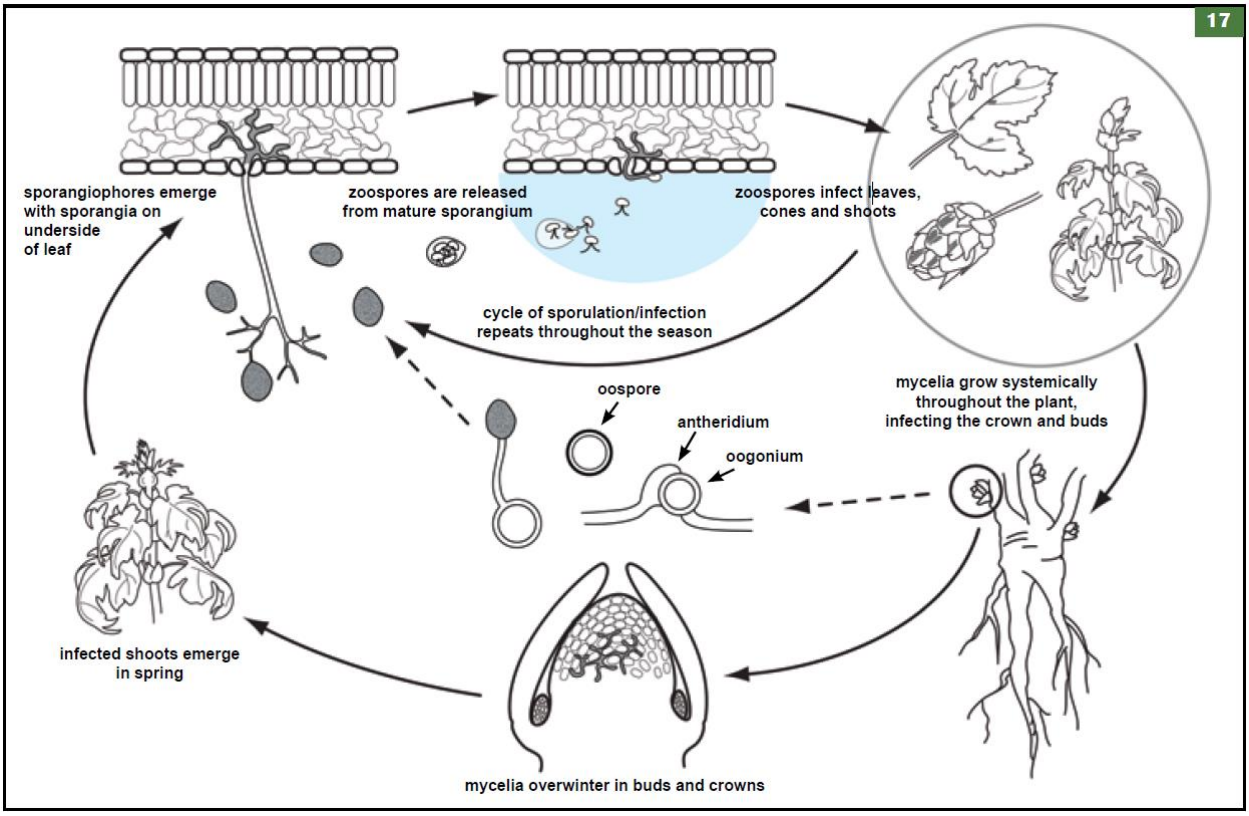

Figure 2: Downy Mildew Life Cycle (O'Neal et al., 2015)

Symptoms

DM affects yield by leaf blight and yellowing, curling of leaves, when it causes local infections. This reduces photosynthetic potential. When the disease infects cones, it causes browning, reduction in size, reduced lupulin gland expression, and reduced oils and bittering components, thus reducing the quality of the cone. If the disease becomes systemic, meaning it overwinters in the crown buds, stunted basal shoots and yellowish appearance to the terminal buds and sidearms. Symptoms are likely to spread when temperatures are between 15.5°C to

21.1°C, and when there is at least 1.5 hours of free moisture on plant surfaces from rain or dew (Hausbeck et al., 2016).

Inheritance of DM Resistance in Hop

DM resistance is reported to be quantitative and polygenic (Henning et al., 2015). This is advantageous because resistance is more robust and more difficult for the pathogen to overcome. However, it means that molecular breeding efforts are more challenging compared to simple gene forms of disease resistance. This contrasts with Powdery Mildew (*Podosphaera macularis*), which is thought to have a qualitative (single gene) resistance mechanism (Henning et al., 2015). Henning et al. (2015) mentions that narrow sense heritability for resistance to DM is $h^2 = 0.49$. This suggests that DM resistance is partially due to non-genetic factors.

Hop Breeding

Breeding Objectives

Initial breeding efforts were focused on increasing resin content, which increased bitterness and brewing efficiency. Additionally, breeders started selecting for increased resistance to diseases and pests. Other breeding program objectives included increased yield, new aroma varieties, adaptation to varying daylength, and dwarf variety development especially in the United Kingdom (Darby, 2005).

The objectives for the breeding program proposed in this creative component (CC) are as follows. Cascade was initially chosen as one of the parents in the proposed breeding program explained in this CC due to a personal observation of DM in this cultivar. The initial goal was set to improve the resistance to DM, while maintaining the flavor and aroma components that

make Cascade such a popular cultivar within the brewing industry. While Cascade is reported to offer moderate resistance to DM (Hop Growers of America, 2015), it still suffers from DM by yield and quality reductions.

Conventional Breeding

Only female plants are grown for cones, so male plants are not readily available. Several sources of male hop plants are available to hop breeders. One source is accessions in the USDA National Clonal Germplasm Repository (NCGR) in Corvallis, OR. Another source is purchased seeds grown out to plants to determine sex. Third, one could find wild growing male hops. Lastly, male flowers can be found on monoecious hop plants which bear both male and female flowers on the same plant, either due to stress or genetics (chromosomal imbalance) (Matthews et al., 2013). Open pollination occurs readily when male and female plants are adjacent to one another.

The proposed breeding program is modeled on a breeding scheme presented by Dr. Peter Darby at the 2018 Hop and Barley Conference (Darby, 2018).

Population Development

The first step in developing a new hop breeding program is development of a segregating population. Sufficient genetic variability needs to be available to make successful selections. Hop plants are highly heterozygous and thus recovering a recurrent parent by way of backcrossing would be unlikely. An F_1 population that is created from cross-pollination of two un-related parents will be highly heterozygous as well. An alternative method could be to allow multiple male hop plants to pollinate the same female hop plant. For this breeding program, Cascade was selected as female parent due to partial resistance to DM and because the objective of the breeding program is to develop a cultivar with similar aroma and flavor profile

as Cascade, but with improved DM resistance. A DM resistant male is desirable as pollen donor, and can be obtained from USDA NCGR in Corvallis, OR. This male plant should not be closely related to Cascade, to prevent any inbreeding depression, and to create substantial genetic variation from which to make selections.

Male and female parents do not have to be adjacent to one another, due to the viability and stability of dry pollen, which can be stored for several months following collection. To ensure pollen purity, hand pollinations are used for cross breeding purposes. Sidearms should be bagged several days before the first florets of the female flowers are receptive to pollen. Sidearm bagging is done by removing several sidearms and leaves, to facilitate placing the bag over the remaining sidearms. Each sidearm will have around 100 inflorescences, with 20 or more florets. One sidearm will produce between 200 to 400 seeds following a single pollination. When florets become receptive, the pollen is introduced inside the bag on the female plant when the bracts are flowering to complete the pollination. Several pollinations may be made several days apart. This is because florets become receptive to pollen over the course of about a week. Pollinations can be made by inserting a pollen filled syringe into the bag, depositing the pollen in the bag, and closing the hole with tape. Next, the bag is agitated by shaking to spread the pollen over the receptive florets. About 50 days after pollination, seeds are mature and can be collected in September. Cones are dried and seeds are rubbed out by hand. Sieves are used to separate seeds from cone and plant material (Haunold, 1980).

Clonal Selection

Hop seeds are seeded into pots or greenhouse flats. Hop seeds germinate best after an exposure to cold temperatures for a period of time. This may be achieved by placing pots or flats outdoors during winter months, or by cold temperature pre-treatment.

With the goal to grow 15,000 F_1 seedlings, and assuming an 80% germination rate (Haunold and Zimmerman, 1974), 18,750 seeds need to be collected. Assuming 300 seeds per sidearm on average, at least 63 sidearms need to be pollinated and harvested. This will form our starting population, and provides the largest amount of genetic variance in the breeding cycle.

Once 15,000 seedlings are established in the glasshouse, they can be screened for DM by inoculation with zoospores. Zoospores are suspended in distilled water and applied with a spray bottle on leaf tissue when seedlings are approximately 10 cm tall (Darby, 2018). The greenhouse is kept at high humidity to favor infection. Plants without symptoms are transferred to the hopyard the following spring into single hill plots.

Those approximately 1000 F_1 plants can be spaced more closely than standard hopyard spacing, which favors disease pressure and thus helps to identify and select resistant plants. During this time, plants will be evaluated for growth habit, pest and disease resistance, cone habit, and sex. Initial observations on aroma and alpha acid concentration can begin as well.

Next, the best 10% are selected and propagated to two environments in multi-hill plots. Here, they can be evaluated for oil components, yield estimates, with continued focus on growth habit and resistance to disease and pests. Clonal selection will occur in the first four years.

Field Testing

In years 5-7, the number of environments is expanded to five locations on larger multi-hill plots (10 to 20 hills/plot). 30 selections will make it to this step. At this stage, pilot brews can be done to explore unique flavor characteristics and aroma profiles compared to the aroma profile of Cascade.

After year 7, the top 3 or 4 experimental lines will be selected and moved to commercial scale hopyards of about 0.4 to 0.8 hectares. In the commercial setting, brewers can evaluate the potential candidates more thoroughly. This evaluation includes strong tolerance to mechanical harvest, drying, and storage. High yield, disease resistance, and ease of training to climb the hopyard strings will be scrutinized as well. Best clones are candidates for registration and commercialization on a broad scale.

Cultivar Release

Following approximately four years of on-farm testing in a commercial setting, top performing clones can start the registration process to become new cultivars. A virus-free stock will need to be established. Virus-free clones are maintained by the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) at the Northwest Plant Germplasm Repository in Corvallis, OR (Haunold, et al., 1984).

Conventional Hop Breeding Program Outline

Year 1:	Cross Cascade x DM Resistant Male—Collect F1 Seeds in the Fall Greenhouse Seedling Disease Screen in Winter (Downy Mildew)—15,000 Hop Seedlings
Year 2-3:	Plant seedlings in individual hills Observe to determine sex, cone habit, general pest and disease resistance, productivity—select 1,000 plants
Year 4:	Propagation to multiple hill plots in 2-3 locations Oil Components Analysis 100 plots/location
Year 5-7:	Collect Commercial Information (Yield, Oil Yield) at 3-5 locations Start Pilot Brews 30 plots/location
Year 8-12:	Commercial Farm Trials and Commercial Size Brewing at 10 locations 3 cultivars per location
Year 12+:	Registration of New Variety

Figure 3: Conventional Hop Breeding Program Outline

Biotechnology

While there do not appear to be any breeding programs in the United States utilizing genetic breeding tools to insert or delete specific genes, there are various applications of marker-assisted selection (MAS) globally (Cerenak et al., 2005; Henning et al., 2015). Specifically, amplified fragmented length polymorphisms (AFLP) segregation analysis was used by the Slovenian Institute for Hop Research and Brewing to correlate molecular markers to genes for conferring resistance to damson hop-aphid (Cerenak et al., 2005). SNP markers were used in hops for genome fingerprinting of cultivars and for the identification of QTL linked to resistance to DM by high-density genetic mapping (Henning et al., 2015; Henning et al., 2015). A draft genome was published by Natsume et al. (2014). In 2017, HopBase, an online resource that contains the current hop genome sequences of two cultivars, ‘Teamaker’ and ‘Shinsuwase’, and a male accession, USDA 21422M was released by Oregon State University

as the first genomic database of *H. lumulus* (Priyam, 2015). This resource should enable greater application of molecular breeding methods in hops (Priyam, 2015).

While resistance to DM in hop is thought to be quantitative, there are examples of resistance in related species that can be explored for future introgression into the hop genome.

Arabidopsis thaliana is a plant often used as a crop model due to its relatively simple genome, which has had its genome sequenced and has phenotypes associated with many genes more thoroughly than most other crops. One gene DMR6 (downy mildew resistance 6) has been shown to reduce the incidence of downy mildew infection in Arabidopsis (See Appendix A for DMR6 sequence). Basic Local Alignment Search Tool (BLAST) is a method of querying a particular genome database to search for a particular nucleotide or protein sequence. When the gene sequence of DMR6 was BLASTed using HopBase, a match for the protein sequence was found in Shinsuwase with an E-score of 0 (Figure 4). An E-score of 0 indicates that this match is not due to chance, implying a high level of sequence homology. In order to use this sequence in hop we have to validate it. This can be achieved by conducting a gene-based association study, testing a population of a genetically diverse hop plants segregating for DM resistance and determine how strongly it associates with markers for DMR6 (Henning et al., 2015). First, we would have to create markers that identify the DMR6 gene. Next, we would screen seedlings for DM resistance. If the DMR6 gene is successful at conferring DM resistance, then we should see a strong correlation between DMR6 and DM resistance within the population.

gnl|BL_ORD_ID|37329 HL.SW.v1.0.G001785.1 1 / 102

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Figure 4: BLAST results comparing DMR6 in A. thaliana to H. lupulus.

According to Woods and Gent (2016), resistance to DM in hop is quantitative and polygenic. No major gene has been identified for resistance to DM in hop. For this reason, MAS may be beneficial to improving genetic gain. If SNPs or other molecular markers can be used to fingerprint seedlings for broad spectrum resistance to the pathogen, some efficiencies may be gained using Marker Assisted Selection to improve selection intensity (Henning et al., 2015). Henning et al. (2015), used a bi-parental mapping population to associate SNP markers with a response to DM infection. The population consisted of 91 offspring from a cross between 'Teamaker' and USDA-ARS 21422M. Mixed linear model analysis identified 39 SNP markers, from which four SNP markers were identified as highly selective for resistance to DM, using high resolution melting curve analysis (HRM). These four SNP markers explained 43.7% of experimental variation. Henning's group will do more validation using different populations before setting up a panel to identify resistance to DM using four SNP markers. This would

enable hop breeders combine greenhouse-based phenotyping with a SNP marker panel to select for a wider range of environmental conditions.

In a related study, Henning et al. (2015) provides a high-density genetic map, based on 3341 SNP markers. His group then identified a total of 22 QTL for DM resistance in a population of 85 offspring created from a cross of 'Teamaker' and 21422M. The 22 QTL were distributed across eight linkage groups (LG) out of 11 total, with greatest densities at chromosome 1, 2 and 5 for field-based DM resistance. Three environments were evaluated, Oregon, Washington, and a Greenhouse. Most of the QTL showed significant additive and dominance effects. No epistatic interactions between QTL were detected. The R^2 for OR was 0.64, for the greenhouse R^2 was 0.65 and for WA, R^2 was 0.93. R^2 was highest in WA likely due to the high disease pressure compared with the other two environments (Henning et al., 2015). R^2 is the correlation coefficient, which measures variation explained by the linear model (Minitab Blog Editor, 2013). An R2 of 0.93 is a strong correlation coefficient, indicating a strong relationship between the QTL and DM resistance. Further validation is required before these QTL can be used with great confidence. Focusing on specific areas of the genome that are likely to contain QTL for DM resistance using SNP markers may provide a good way to make selections.

Another option that could be used to improve genetic gain in hop breeding is genomic selection (GS) (Crossa et al., 2017). However, due to the time and resources that go into developing and maintaining a training population, this would not currently be feasible.

A third option for future hop breeding is the CRISPR/Cas9 gene editing technology (Reis, 2014). CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats, discovered in bacteria as a form of immune system, used to store bits of viral DNA, in order to recognize and eliminate any future encounters with such a virus. If a similar virus is

encountered, a guide RNA strand will be created and bind with the virus, allowing the virus DNA to be cut to disable it. This mechanism has been modified to be used in most living cells. This allows researchers to edit DNA sequences with greater precision than before (Gao, 2018). CRISPR/Cas9 could be used, for example, to insert a DNA sequence that was described earlier as DMR6, in order to test whether this gene provides any DM resistance in hop. Much work has to be done before such transformations would be possible. For native DM resistance in hop, which is quantitative, many genes would have to be inserted. CRISPR/Cas9 is better suited for simple gene transformations, such as the gene for Powdery Mildew resistance.

Conventional Breeding Program Cost

Currently, conventional breeding is the main hop breeding method used in the United States. In order to successfully breed hops, a breeder has numerous costs that include land rental, greenhouse, equipment, and time/labor (Table 1).

Table 1: Annual cost for conventional hop breeding program

Input	**Costs**
Land Rental	$741/hectare/year= $9,000
Standard Hopyard Supplies and Installation	$34,250/hectare (2 hectares needed) = $68,500
Greenhouse and Supplies	$7,500
Labor	$15,000/year
Hop Analytics (Hop Profile)	$115/test x100= $11,500
TOTAL ANNUAL COST	**$111,500**

Marketability

A cultivar which exhibits similar flavor and aroma characteristics as Cascade that is more affordable to produce, due to decreased fungicide applications, should be welcomed into the market. It appears that hop breeding is shifting towards the private sector instead of University/USDA efforts. Dr. Peter Darby's breeding program, Wye Hops Ltd., which started at Wye College, was privatized in 2007 as part of the British Hop Association after funding was cut at the college (British Hop Association, 2015). In the United States, Great Lakes Hops, located in Michigan, has its own breeding efforts. Focused on developing new flavor and aroma components, this company has released two proprietary cultivars (Great Lakes Hops, 2018). In contrast, USDA-ARS released its most recent cultivar in 2006, with the introduction of 'Teamaker'. Both efforts show that the hop and beer market favor not only the introduction of new cultivars, but also cultivars that are resistant to diseases, ultimately allowing companies to increase profit. An example of the revenue to be earned from a new cultivar is 'Mosaic' or HBC 369. Breeding for HBC 369 started in 2001, and the cultivar was released in 2012. After four years of commercial production, about $2,316,600 can be earned from sales of Mosaic rhizomes (Table 2). Table 2 assumes 2200 hills per hectare, and a price of $4.50 per rhizome (Sirrine et al., 2014). HBC 369 is Plant Variety Protected (PVP), meaning only Hop Breeding Company LLC (HBC) can sell HBC 369 rhizomes (Carr, 2017). In 2017, HBC 369 represented about 1% of harvested hop acres in the U.S., which would be considered quite successful for a new cultivar (National Agricultural Statistical Service, 2017). If we assume a ten-year development cycle at $111,500 per year (Table 1), cost comes to $1,115,000 with revenue at $2,316,600 five years after introduction. Net profit is revenue minus cost, or $1,201,600. This means profit from HBC 369 is about double its initial investment.

Table 2: Revenue from rhizome sales for Mosaic

Year	**Hectares**	**Increase ha from previous year**	**Revenue**
2012	0	--	0
2013	0	--	0
2014	32 ha	32 ha	$316,800
2015	71 ha	39 ha	$386,100
2016	205 ha	134 ha	$1,326,600
2017	234 ha	29 ha	$287,100
Total		**234 ha**	**$2,316,600**

(Source of data: National Agricultural Statistical Service, 2017; Sirrine et al., 2014)

Future Use of Biotechnology in Hop Breeding

With the introduction of the HopBase database and decreasing costs of genome editing tools, these tools may increasingly become implemented in hop breeding. A more robust hop genome database would be beneficial. The database would be strengthened by including a greater number of fully sequenced cultivars and wild-types. Marker assisted selection will continue to play an important role in hop breeding. In order to adapt to cultural and chemical control practices, marker assisted selection is going to need to play a vital role in the industry. While it may be difficult to increase the cycles per year with conventional breeding, there may be opportunity to increase selection intensity by utilizing MAS to improve genetic gain. This may

be done by GS. Once a training population exists, selections may be made at the seedling stage, allowing for a larger starting population, given the same number or resources. Additionally, it would be possible to assign breeding values to male plants, to improve parental selection for hop breeding.

Cost may yet be prohibitive for using genome editing techniques, and more research needs to be done to isolate the QTL or specific genes responsible for DM resistance. Hop breeders may consider a reduced emphasis on alpha acid concentration. Dr. Peter Darby suspects an inverse relationship between alpha acid concentration and disease tolerance, or intense selection for one attribute leading to lower performance of other characteristics (Darby, 2018).

Appendix A

Protein sequence for DMR6 in *Arabidopsis thaliana* (FASTA format):

```
>sp|Q9FLV0|DMR6_ARATH Protein DOWNY MILDEW RESISTANCE 6 OS=Arabidopsis
thaliana OX=3702 GN=DMR6 PE=1 SV=1
MAAKLISTGFRHTTLPENYVRPISDRPRLSEVSQLEDFPLIDLSSTDRSFLIQQIHQACA
RFGFFQVINHGVNKQIIDEMVSVAREFFSMSMEEKMKLYSDDPTKTTRLSTSFNVKKEEV
NNWRDYLRLHCYPIHKYVNEWPSNPPSFKEIVSKYSREVREVGFKIEELISESLGLEKDY
MKKVLGEQGQHMAVNYYPPCPEPELTYGLPAHTDPNALTILLQDTTVCGLQILIDGQWFA
VNPHPDAFVINIGDQLQALSNGVYKSVWHRAVTNTENPRLSVASFLCPADCAVMSPAKPL
WEAEDDETKPVYKDFTYAEYYKKFWSRNLDQEHCLENFLNN
```

Source: UniProt. Protein sequence for DMR6 in *Arabidopsis thaliana* (FASTA format). 2018. http://www.uniprot.org/uniprot/Q9FLV0

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