Investigating the Distribution and Diversity of *Leptosphaeria maculans* in Northern Idaho

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Authorization to Submit Thesis

This thesis of Justin E. Pickard, submitted for the degree of Master of Science with a Major in Plant Science and titled “Investigating the Distribution and Diversity of *Leptosphaeria maculans* in Northern Idaho," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Most of the oilseed production in Idaho takes place in the panhandle, ranging from the Canadian border to the Camas Prairie in north Idaho county. This region of the Pacific Northwest has a Mediterranean-like climate, with cold wet winters and hot dry summers. Dryland cropping systems in the Palouse region plant both winter and spring varieties of canola (*Brassica rapa, B. juncea, B. napus)*, where the product is either sold for food grade oils, bio-fuel crushing, or seed production. The lack of vegetable production in north Idaho allows for producers to grow canola as a certified seed crop, increasing value dramatically. In 2014, a grower producing industrial rapeseed in Lewiston, ID had samples intended for certified seed test positive for the black leg pathogen, *Leptosphaeria maculans*. Black leg is the most serious disease of canola worldwide and has resulted in significant epidemics in many major canola producing regions. This disease was first recognized in northern Idaho in 2011 and may pose a risk to the northern Idaho canola industry since disease resistance within local winter canola varieties is largely unknown and growers are not well educated in proper management strategies. Although disease symptoms have been minor to date, with little progression into the upper canopy and very few stem cankers, the frequency of infection is widespread. From 2016-2017 a survey of 50 oilseed fields in northern Idaho, 38 were found to display symptoms of blackleg. Isolates of *L. maculans* (127) and *L. biglobosa* (10) were recovered from these infected fields. Analysis of *L. maculans* isolates revealed a near equal distribution of mating types, suggesting widespread distribution of ascospores and sexual recombination. Utilizing known plant resistance differentials and PCR, the diversity and distribution of avirulence genes was determined. The highest frequency avirulence genes observed in north Idaho were: *AvrLm 5,6,7,11*; *AvrLep R1*, *R2*. When the results were

combined to create geographic races, only six races had five or more isolates. The most common race in north Idaho was *AvrLm 5-6-7-11-LepR1-LepR2¸* which was found in 20% of all isolates sampled. By characterizing local populations of *L. maculans*, information gained from this project will be useful in identifying or developing canola germplasm with the appropriate genetic resistance for use in disease management in northern Idaho.

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Dedication

To many of my friends and family who never really understood what I was doing or why,

your unconditional love is enough.

Without, I would have forgotten the importance of life outside of work.

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CHAPTER 1: LITERATURE REVIEW

1.1 Dryland Cropping Systems in the Pacific Northwest

The Inland Pacific Northwest (iPNW) has a Mediterranean-like climate with cool, wet winters and warm, dry summers. Average annual precipitation ranges from 60 cm on the eastern edge (Washington-Idaho Panhandle border) to less the 15 cm on the west edge (Columbia Basin) (Kok et al. 2009). Common topography in the Palouse region of the PNW is steep, with commonly farmed slopes ranging from 30%-45%. This steep topography often occurs across the PNW in intermediate to high precipitation zones where most of the annual cropping systems are found (Kok 2009; USDA 1978).

Historically, the iPNW crop rotations have been winter wheat cropping systems that incorporate the use of tillage to prepare the seed bed before planting. A typical 3-year crop rotation in northern Idaho is winter wheat, spring cereals, and legumes. Spring canola is often added into 6-year crop rotations, but also commonly takes the place of spring cereals when more profitable or of legumes when soil conditions are not suitable for legume production (Jim Davis, personal communication). This results from common problems in the region with soil acidity, resulting from decades of using ammonium-based nitrogen fertilizers (Koenig et al 2011). When low pH soils occur, the ability of legumes to fix nitrogen is diminished and the yield is reduced (Graham and Vance 2014). Consequently, farmers may drop legumes from their rotations when the extra nitrogen produced by the crop is no longer cost effective. Canola is most commonly grown following spring cereal crops, making it an easy supplement for poor performing legumes (Brown et al. 2008). Adding additional crops to a diverse

cropping system can reduce disease risk for barley, wheat, and legumes, but successive planting of canola should be avoided to limit *Brassica* pathogens (Johnston et al. 2005).

Spring canola can be planted in dryland conditions without a fallow period, making it easier to add to an existing rotation, while winter canola relies on a fallow rotation beforehand to store ground moisture (Brown et al. 2008). Winter crops are predominantly grown to take advantage of higher yield. Winter canola often supplements intensive 2-year winter cereal and fallow production rotations by providing an additional crop to fallow, changing production to a 4 year rotation of winter wheat, fallow, winter canola, fallow (Jim Davis, personal communication).

Over the last four decades, efforts have been made to shift conventional production systems to conservation and no-till systems to reduce erosion (Kok et al. 2009). On the Palouse region 40% of topsoil has been lost in the last century alone (Pimentel et al. 1995). Conservation and no-till systems aim to reduce erosion by lessening the damage caused to the top layer of soil. Conservation tillage achieves this by reducing the amount of tillage operations a farmer completes in the field to produce the next crop. In conventional tillage systems, the typical number of operations between winter wheat and a spring crop was eight during the 1970's. Reducing the number of tillage operations to 1 and using new equipment combining fertilization with planting, modern conservation systems in the north Idaho region can achieve 2 or 3 operations between winter wheat and a spring crop (Kok et al. 2009). No-till, also referred to as zero-till, is a system that takes advantage of existing ground cover left over after harvest. Aside from pest or weed control, the only disturbance to the field occurs during planting, which helps with soil composition as multiple crops are rotated though the field over time. Benefits from no till include: winter crop insulation, higher moisture

retention, enhanced soil composition, and dramatically reduced erosion. Typically crops with smaller seeds have issues related to planting in no-till fields. When canola is direct seeded into straw, seedling development can be reduced due to ground temperature and poor soil contact. (Brown et al. 2008).

1.2 Canola Production in North Idaho

Canola refers to a *Brassica* crop that produces seed oil maintaining less than 2% uricic acid and whose crushed oil-free meal contains less than 30 μmol of glucosinulates (Brown et al. 2008). The evaluation of oilseed and vegetable *Brassica* crops can be derived from three diploid species (*Brassica rapa, B. oleracea, B. nigra*) whose inter-crossing resulted in an additional three allotetraploid species (*B. carinata, B. juncea, B. napus*). This relationship was described in 1935 by Nagaharu U and is commonly referred to as the "Triangle of U" (U 1980).

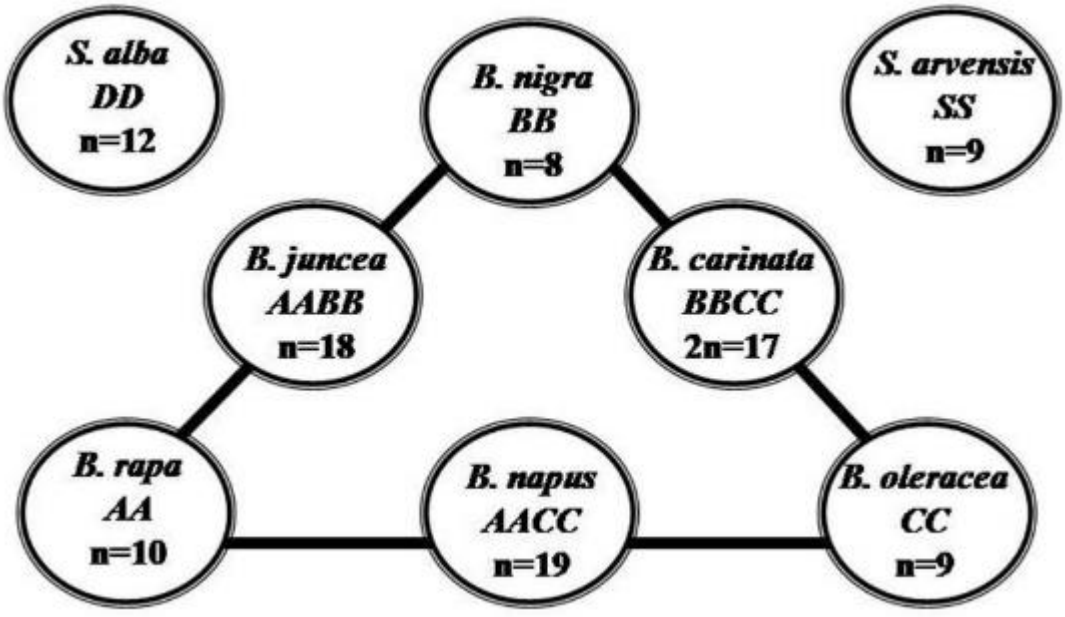

Figure 1.1: Cross pollination relationship described as the "Triangle of U," (Brown et al. 2008, adapted from U, 1936)

There are three species of canola grown in northern Idaho, with the most popular being *B. napus*. Its yield potential is higher than other species of *Brassica* crops, making it popular with plant breeders who aim to provide high yielding canola crops. *Brassica rapa*, has limited amounts of spring and winter cultivars on the market. It is often planted to take advantage of its earlier bloom period and reduced pod shatter, but acreage has dwindled in comparison to *B. napus*. *Brassica juncea*, commonly referred to as 'Indian Mustard', has limited acres in the PNW and is exclusively a spring crop. Its acreage peaked when it was marketed as one of the first Clearfield varieties (Jim Davis, personal communication). Many *B. juncea* mustard cultivars are grown for bio fumigation and high-glucosinulate meals, making them more popular as alternative crops.

Aside from profitability, canola is often added to rotations for its rotational benefits. Canola's specialized tap-root system helps create space for water infiltration, loosens hard pans, and accesses root zones in the soil that are too deep for cereal crops. Water infiltration impacts fields by reducing water runoff and helps distribute water efficiently, adding soil moisture. Following oilseed crops, wheat can sometimes benefit from this distributed moisture if in supply, helping increase shoot density (Angus et al. 1991). Troublesome hard pans are broken up by canola's aggressive tap-root system, which can grow into the compacted zone and help loosen soil (Jim Davis, personal communication). This deep rooting system penetrates deeper than average fibrous roots of cereal crops, giving reach to additional moisture and leached nitrates from previous growing seasons (Merrill et al. 2002; Weinert et

al. 2002). Consequently, this additional stored ground moisture can be tapped dry if field capacity is not replenished.

Winter and spring canola are both great additions to north Idaho rotations but have different advantages that affect management strategies. Late winter to spring field conditions in north Idaho are typically very wet and can cause issues with spring planting. Planting strategies directly affect yield, with spring canola production in north Idaho favoring early-April planting to mitigate high temperatures during flowering in June. Winter canola has a less sensitive planting date but requires extra ground moisture that can be achieved with a fallow period beforehand (Brown et al. 2008).

Canola is commonly grown following spring cereal crops, with typical complications arising from herbicide carryover in the soil (Brown et al. 2008). Many producers grow Clearfield (IMI) wheat, which relies on the group 2 herbicide Beyond (imazamox). Despite a canola plant-back period of 26 months, it maintains regional popularity for fighting jointed goatgrass (*Aegilops cylindrica*) in winter wheat crops. One of the larger plant-back restrictions for canola occurs after the use of Pursuit (imazetharpyr) on legumes, a group 2 herbicide. Label recommendations do not advocate planting canola for 40 months and suggest producers conduct a yearlong bioassay beforehand. For both of these common herbicides, the only way around plant back restrictions is to use Clearfield varieties of canola, which does not directly address the underlying plant-back restrictions.

Canola can flower and develop seed until stress terminates the development process; consequently early periods of stress can greatly impact seed yield. When compared to wheat, canola is more sensitive to heat stress due to flowering and seed initiation occurring over a long period of time. Due to environmental stresses during seedling establishment and

flowering, canola has a higher yield variability than wheat (Koenig et al. 2011). In canola production, the duration of time plants are in bloom directly relates to yield. In north Idaho, winter canola begins blooming in early May and does not have issues with high temperatures before petal drop. When averaged over years and sites, early planting dates in the beginning of April caused spring canola in north Idaho to flower between June 18th and 23rd. When planting was delayed by two weeks, bloom was delayed by 8 to 10 days (Reed 2015). Conditions affecting bloom period are high temperatures beginning in the end of June, which lead to early petal drop and initiation of pod filling around 29-32°C. Farmers are advised to plant spring canola as soon as ground temperatures are consistently above 7°C in order to avoid germination issues and hard frosts with earlier seeding (Jim Davis, personal communication).

When winter canola is planted too early, young plants are more susceptible to insect damage reducing stand health or get too large and deplete available soil moisture for the subsequent growing season, limiting plant development (Brown et al. 2008). When seeded too late, plants not reaching the initial rosette stage are very susceptible to winter kill (Jim Davis, personal communication). Winter canola bloom is not directly affected by planting dates if the plant reaches the six-leaf rosette development stage before freezing. Appropriate winter planting dates aim for a minimum of 300 growing degree days (GDD) before the onset of winter conditions. Survival rates are optimum when individual plants reach rosette stage, with at least four fully opened leaves, which takes around 433 GDD (Jim Davis, personal communication). GDD is calculated for canola by subtracting 4°C from daily high temperature and adding the resulting values. This number can be reached easily in 2 weeks when temperatures are high in late August, but as temperatures steadily drop into late-

September the 6-leaf rosette stage could take over a month to reach (Jim Davis, personal communication).

1.3 Production and Economics of Canola

Canola is categorized by the USDA as a field crop. In 2012 a total of 3,995 farms in the US contributed 0.7 million hectares, a 0.1 million acre increase from 2007, and production accounted for 1 million metric tons of harvested canola (USDA-NASS). The largest producer of canola in 2012 was North Dakota, which at 0.6 million hectares, accounted for 83.6% of all canola production in the US (USDA-NASS).

In Idaho, 133 farms grew canola in 2012, increasing by 47 farms from 2007. Acreage nearly doubled in the same 5-year span, with 14,754 hectares grown, accounting for 2% of U.S. production. Harvested quantity nearly tripled, with 28,771 metric tons (USDA-NASS). Idaho's production of canola has declined since then, with 8,296 hectares harvested in 2016, contributing $6.63 million to the state economy at a state average price of $15.4/cwt. By comparison in 2016, Montana produced 24,000 hectares, Oregon produced 1,500 hectares, and Washington produced 12,500 hectares (USDA-NASS).

1.4 Idaho's Oilseed Production Laws

Idaho currently has two rapeseed production districts, previously revised from seven. District 2, which encompasses Payette, Gem, Canyon, Ada, and northern Owyhee county, put a ban on rapeseed production. The ban was put in place to prevent cross contamination between edible and industrial rapeseed, as well as contamination of *Brassica* vegetable crops grown for seed in southern Idaho (IDAPA 2018). District 1 is defined as the rest of Idaho.

Most of Idaho's canola production takes place in the north panhandle, ranging from the Canadian border to the Camas Prairie, north Idaho county (IDAPA 2018).

According to state regulation, before planting industrial rapeseed in Idaho, the producer must make sure that the field is at least 1-mile away from edible rapeseed and obtain written permission from owners of neighboring fields. To plant *Brassica* seeds in Idaho, all seeds must be treated with EPA and State registered fungicide for the control of black leg. In addition, seed that is produced outside of Idaho must be accompanied by a phytosanitary certificate, stating that the seed is free from black leg, meeting a minimum of 1000 tested seeds. If the seed was sold in lots of less than 2 lbs or if the seed was produced in Idaho, than the seed is exempt from these rules (IDAPA 2018).

1.5 Diseases of Canola

Canola is susceptible to a large range of disease which can impact the host from seedling to maturity. In north Idaho, black leg (*Leptosphaeria maculans* and *Leptospharia biglobosa*), *Rhizoctonia* root rot (*Rhizoctonia solani*), and *Sclerotinia* white mold (*Sclerotinia sclerotiorum*) are the most concerning fungal pathogens (Koenig et al 2011). Other species of *Brassica,* including mustard (*Sinapis alba*) and cruciferous weeds, can succumb to the same diseases and act as alternate hosts in cropping systems. Incidence and severity of these diseases depends on farming practices, environmental conditions, and the intensity of crop rotations (Bailey et al. 2003).

The fungal disease Sclerotinia white mold can cause heavy yield loss in canola. Although present in the Pacific Northwest, the disease is not typically serious, while individual fields in North Dakota and Minnesota have shown losses as high as 13 to 50

percent (Markell et al. 2009). Surviving in the soil as sclerotia, excess moisture from spring and summer rains triggers these small black structures to germinate. Emerging from the soil as apothecia, the ascospores can be released during the optimum bloom period of canola. Free water and 100% relative humidity are required for ascospore germination. Ascospores land on flower petals that have dropped, typically stuck in the leaf axil where infection often begins. As the disease progresses, infected regions of the plant will appear bleached (Paulitz et al 2015). Later in the dry season, serious infection can lead to premature death and lodging. Established infections result in hard black structures called sclerotia that remain inside plant tissues as an overwintering mechanism (Markell et al. 2009). Sclerotia can contaminate seed during harvest, leaving behind plenty of inoculum to survive until the next season. As an important control point, seed should be properly cleaned to prevent movement of the pathogen into new areas (Paulitz et al. 2015).

The soil pathogen, *R. solani*, is the primary causal agent of damping-off and root rot in canola crops (Verma 1996). Typically, infected plants are scattered in the field or grouped in low, poorly-draining patches. Symptoms consist of brown to grayish lesions, either smooth or scurfy, with visible development spreading upwards from the stem base (Bailey et al. 2003). Categorized by anastomosis groups (AG), the ability of the pathogen to induce symptoms varies. Isolates belonging to AG2-1 are highly virulent and responsible for pre-and post-emergence damping-off of canola seedlings. Isolates belonging to AG4 mainly attack adult plants causing basal stem rot later in the growing season (Verma 1996).

1.6 Black Leg of Canola

Black leg, also known as stem canker or Phoma stem canker, is attributed to a widespread fungal pathogen that infects plants in the family Brassicaceae. Classified as

Leptosphaeria maculans (anamorph *Phoma lingam*), the fungus causes concerning symptoms on all *Brassicas,* including canola (*B. juncea, B. napus,* and *B. rapa*). With the ability to cause stem canker on both spring and winter cultivars of oilseed rape, *L. maculans* can be found in a wide range of climates, despite widely varying agricultural practices (West et al. 2001).

The pathogen is successful in dry Mediterranean climates which generally have mild winter temperatures and hot summers near the sea or severely cold temperatures in the winter with hot dry summers inland (Kassam et al. 2012; West et al. 2001). The survival of the disease is affected by soil moisture and temperature, which control the rate of residue degradation. Things that slow this rate include dry summers and cold winters, which describe a Mediterranean climate. In Western Australia, residue containing pseudothecia can remain an inoculum source for up to 4 years. While in the wetter southeast region, oilseed rape residues declined in volume by 90% in 1 year, dramatically reducing ascospore production the following season (West et al. 2001). Western Australia, experiencing this climate, faced losses approaching $50 million in 1999 (Khangura et al. 2007). When Australian producers plant oilseed in the wet season, the timing of rainfall coincides with ascospore production. This increase of inoculum during seedling establishment results in early infections, leading to plant death or severe cankers later in the growing season (West et al. 2001).

Canola in north Idaho is grown in a Mediterranean-like climate, with cold wet winters and dry hot summers (Kok et al. 2009). Black leg of canola was recently identified in northern Idaho with the first incidence of verified blackleg in 2011 when a dried stem sample was collected from Bonner’s Ferry, exhibiting dark gray lesions with black pycnidia (Agostini et al. 2013). The source of seed was unconfirmed.

Leptosphaeria maculans attacks cotyledons, leaves, stems, and pods. Leaf lesions are greyish white, round to irregular, and usually dotted with numerous black pycnidia. Poorly defined, white or grey lesions later form on the stems, often starting at the scar of fallen, infected leaf. In wet weather, pycnidia on the stem and leaf lesions exude pink or purple spore masses which can be dispersed short distances by splashing rain (Bailey et al. 2003).

In severely infected plants, stem bases develop dry sunken cankers with a black border. Cankers may completely girdle the stem bases during pod filling, resulting in premature ripening, leading to shriveled seed. In extreme examples of the disease, severe cankers can sever plants at the stem base. Seed can become infected and serve as a source of inoculum in the subsequent crop. When transported post-harvest, infected seeds can be responsible for the introduction of the pathogen into new areas. Pods on plants with cankers often shatter before healthy plants are ready for harvest (Bailey et al. 2003). A study featuring infected seed germination in the greenhouse found infected seed had a lower germination rate (75%) compared to clean seed (98%), with infected seedlings dying from black leg as early as the 3rd leaf stage (Van de Wouw et al. 2016).

Pod infection occurs later in pod development and occurs less frequently than other symptoms, possibly because it is only partially related to initial infection. Chigogora and Hall (1995) correlated severity of stem cankers and pod infection across a whole plot, leading to the conclusion that pod symptoms were latent infections. A difference in results was observed when infection data was collected across individual plants by Van de Wouw (et al. 2016), finding that pod infection may be more closely related to spore release during podding growth stage, rather than growth up through the plant tissue. Latent pod infections could arise from ascospores or pycnidiospores, but is unknown at this point (Van de Wouw et al. 2016).

1.7 The Black Leg Complex

In the past, blackleg isolates had been classified as two groups. The groups were defined in a number of ways, but typically consisted of an aggressively virulent strain with potential for severe symptoms (A group, Tox^+ group) and a less virulent strain with milder symptoms (B group, Tox^0 group) (Williams and Fitt 1999). When grown in culture and analyzed, each group produced consistent, separate esterase profiles. These two observed esterase profiles were associated with two groups, pathogenic/aggressive (Tox+) and weakly pathogenic/non-aggressive blackleg isolates (Tox^0). Toxicity being defined by the presence of sirodesmin PL, with the highly virulent Tox^+ group containing the toxin and the weakly virulent Tox^0 lacking (Balesdent et al. 1992).

These groups were classified as two fungal species, *Leptosphaeria maculans* being comprised of the more aggressive isolates and *Leptosphaeria biglobosa* encompassing the less aggressive isolates (Shoemaker and Brun 2001). This reclassification was spurred by evidence of plant assays, observations of cultural characteristics and isozyme polymorphism, secondary metabolite profiles, and variability in genome suggesting that black leg could be two groups of organisms (Williams and Fitt 1999). With the advancement of PCR techniques, it is now known that *L. maculans* and *L. biglobosa* are genetically distinct organisms which can be identified and differentiated by amplifying the ITS region (ITS1-5.8S-ITS2) of rDNA. Where the species *L. maculans* is attributed to samples with an ITS size of 468 to 499 bp, and *L. biglobosa* attributed to those with a larger ITS range of up to 550 bp (Mendes-Pereira et al. 2003; Van de Wouw et al. 2008).

Both *L. maculans* and *L. biglobosa* cause symptoms associated with blackleg, but the latter often poses little to no threat to the host plant. The two pathogens are often found

together in the same field or even within the tissues of the same individual host (Vincenot et al. 2008). The main morphological difference between *L. maculans* and *L. biglobosa* is the shape of pseudothecia. In *L. biglobosa*, the wall cells of the pseudothecia inflate causing a long protrusion. The protrusion (papilla) presides on the top of the structure resembling an elongated beak, inflated at the apex. In *L. maculans*, this protrusion is non-existent and the pseudothecia are described as globoid with a barely detectable papilla. (Shoemaker and Brun 2001).

1.8 Pathogen Infection and Colonization

The polycyclic life cycle of *L. maculans* involves both asexual and sexual spore states with several sources of potential inoculum including conidia, ascospores, and infected seed or debris (Figure 2). Survival of the pathogen occurs via infected seed, pseudothecia on crop residue, and mycelium overwintering in previously colonized stem tissue (Bailey et al. 2003). Concerning spore production, there are four key developmental stages: pycnidia, immature pseudothecia, pseudothecia with developing asci, and mature pseudothecia with developed asci containing ascospores (Khangura et al. 2007).

When humidity and temperature reach suitable conditions, pseudothecia mature and produce sexual ascospores. In France, the maturation occurred between 63 and 75 days after infection. (Lô-Pelzer et al. 2009). Pseudothecia in Australia mature between 99 and 171 days after infection, with earlier maturation observed in higher rainfall areas, while drier environments had slower development with longer surviving pseudothecia (Khangura et al. 2007).

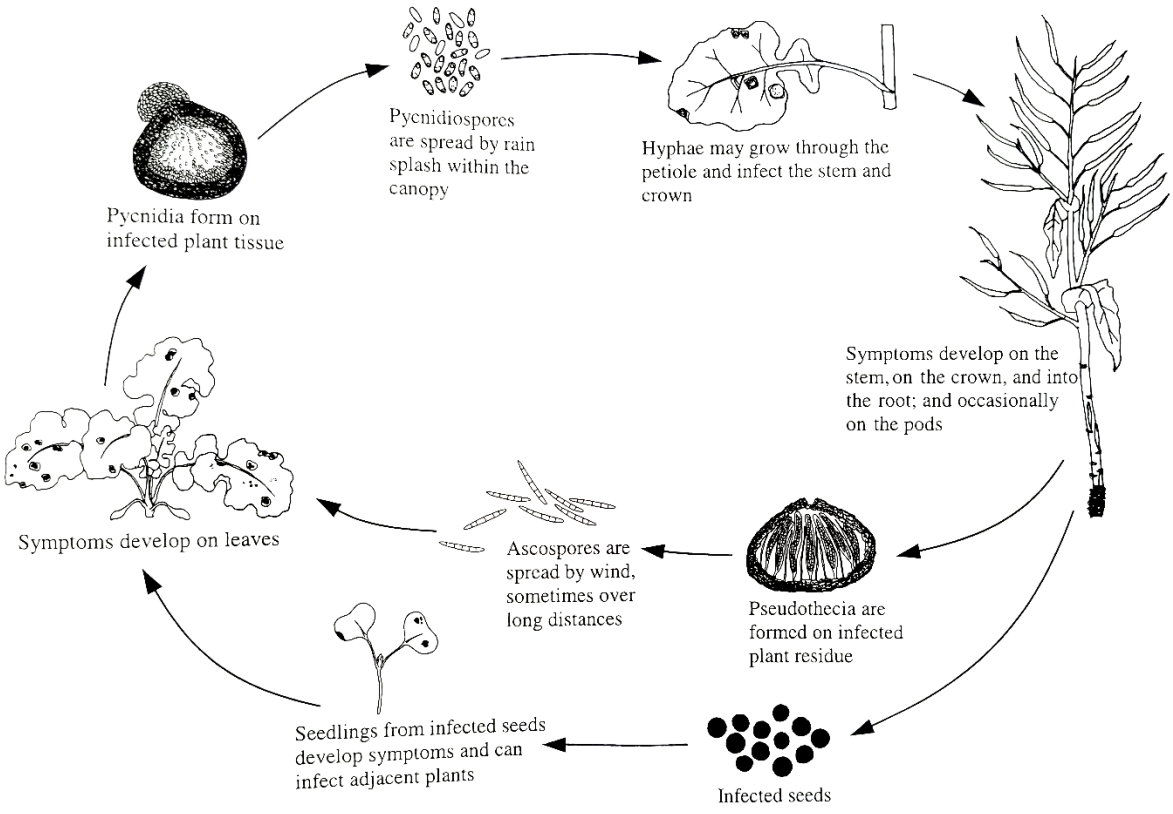

Figure 1.2: Polycyclic lifecycle of *Leptosphaeria maculans* (Buchwaldt 2007).

Once asci mature, ascospores begin to discharge from the pseudothecia residing on crop residue. Timing of discharge can vary, with daily fluctuations based on available moisture and available ascospores. In Australia, a rainfall event of >1.0 mm is required to trigger a large ascospore release, while smaller releases can still occur with light rain or heavy dew. The discharge of ascospores can occur during and several hours after rainfall, dispersing up to 25 m away from the inoculum source (Khangura et al. 2007; Guo and Fernando 2005). Dispersed during optimal periods of wind, ascospores depend on diurnal release patterns to release during the perfect time. The transition from day to night creates temperature fluxes that trigger the release of ascospores in to changing winds (Savage et al. 2013).

Timing of spore release differs between Canada, Australia, and England as shown by simulation work. Often displaying diurnal patterns of release, the discharge pattern may be under regional selection pressure considering its use for organism survival (Savage et al. 2013). Timing of spore release varies by region, resulting in infected plants that are of varying ages. Factors that influence spore dispersal include rainfall, average temperature, and traditional planting dates for *Brassica* crops. Earlier infections that coincide with germination cause more severe symptoms later in the season, like epidemics seen in Western Australia (West et al. 2001).

Discharged ascospores carried by wind, land on leaves and subsequently germinate 2 to 8 h after leaf contact. Lacking appressoria for infection, germ tubes grow randomly along the surface of the leaf, gaining entry to host tissue by open stomata or wounds in as quickly as 4 h. Tissue colonization then leads to tissue collapse and pycnidia production, resulting in observable leaf lesions (Li et al. 2004).

Once a plant is infected, secondary spread of the pathogen can occur via asexual spore stage. Pycnidia develop on the surface of lesions and produce hyaline pycnidiospores suspended in a bright pink or purple matrix. Pycnidiospore production is triggered by high moisture conditions, including relative humidity, dew, and rainfall events. Dislodged up to a meter by rain splash or by wind, the pycnidiospores can travel up to 45 m from the source of inoculum (Guo and Fernando 2005). Secondary infections caused by pycnidiospores take a few days longer to occur than ascospores and will have a lower germination rate overall (Li et al. 2004). The impact on plant health and yield from secondary infections is negligible but can act as a source of inoculum the following year due to increased amounts of infected debris.

Secondary infections may be the cause of pod spot and seed infection, rather than the systemic spread of the pathogen (Van de Wouw et al. 2016).

The colonization stage of the pathogen starts at the infection site and is initially biotrophic with no symptoms. After host tissue penetration, hyphae grow into the intercellular spaces of the mesophyll tissue, developing a mycelial network (Li et al. 2004). After hyphae spreads through the spongy tissue, necrotic cells are left in the wake, resulting in a typical necrotic lesion on the surface of the leaf. Using the vascular system to its advantage, the pathogen colonizes the petiole, spreading within xylem vessels (Hammond et al. 1985).

Until reaching the stem, macroscopic symptoms are unobservable aside from leaf lesions. Beginning the necrotrophic phase, colonizing hyphae kill cells at the forefront of their spread. Damage of the stem cortex below the axil of the leaf begins, where the initial lesions develop, as adjacent cell death spreads (Hammond et al. 1985). Progression of the stem lesion over time further develops into large necrotic regions, described either as cankers or stem lesions. Both can limit the flow of water and nutrients through plant tissue, slowing development and reducing yield (West et al. 2001).

1.9 Disease Management

1.9.1 Seed Certification and Inspection

Leptosphaeria maculans can be distributed long distances on seed and this seed can serve as a means of introducing the pathogen into new regions previously free of black leg (Mahuku et al. 1997). Typically, infected seed is much smaller and is removed by sieves during the seed cleaning process (Lloyd 1959). The viability of seed infected with *L. maculans* dramatically decreases during a 9-month time frame following harvest, which may

help reduce the frequency of epidemics via export. Given that the disease infects and subsists on seed, inspection and certification of seed crops is a necessary measure to prevent transfer of disease. Implementation not only seeks to prevent local spread of the disease, but also intercontinental spread to oilseed producing countries who have not yet tested positive for black leg (Van de Wouw et al. 2016).

1.9.2 Fungicides

Early initial infections are crucial for the development of severe black leg symptoms. Already required as a control for black leg in Idaho, fungicidal seed treatment of canola can reduce initial infections and promote better plant establishment (IDAPA 2018). Seed treatments utilizing iprodione and prochloraz effectively suppress seedborne fungus. When measured in the field, plants were protected for 15 days after germination (Kharbanda 1992). The use of seed treatments greatly benefits producers by eliminating the chance that seedborne fungus is transferred to the field. Although important to control the spread of disease, the use of seed treatments will not protect against early season infections.

Fungicides are an effective strategy when used as part of an integrated pest management solution to reduce initial infections by *L. maculans*. When an azoxystrobin fungicide was applied to susceptible and resistant cultivars at the 2 to 6 leaf stage to control black leg, it did not benefit resistant cultivars and showed a small yield improvement on susceptible cultivars. Given the added cost of fungicide application, this control method is not profitable for canola producers (Kutcher et al. 2013).

A big issue in controlling black leg is the persistence of inoculum-producing stubble in the field (Kharbanda 1992). Stubble left in surrounding fields will carry sporulating

pseudothecia and release inoculum over a season (McCredden et al. 2017). The effectiveness of foliar applied fungicides is only a short-term solution, failing to protect the host from inoculum over a full growing season. A regime consisting of three applications of pyraclostrobin was deemed effective in reducing the severity of black leg symptoms. Given how susceptible *L. maculans* is to selective pressure, repeated applications of the same fungicide may reduce effectiveness over multiple seasons. The rotation of fungicides with different modes of actions is suggested to mitigate the development of fungicide resistance (Hwang et al. 2016).

1.9.3 Crop Rotation

By relying on infected seed, pseudothecia on crop residue, or mycelium overwintering in previously colonized stem tissue, *L. maculans* can survive between growing seasons (Bailey et al. 2003). When grown in continuous monoculture or short rotations, canola residue does not have time to decompose before the next host crop. As a consequence, *L. maculans* can complete and repeat its lifecycle, resulting in successive generations, increased pathogen population, and faster host resistance breakdown (Kutcher et al. 2013). Adding a variety of non-*Brassica* crops in a rotation lowers black leg incidence and severity, reducing disease pressure caused by continuous cultivation. The use of alternative crop rotations gives residue time to break down while limiting the population of host plants. A simple rotation of canola and wheat significantly reduced disease by 18% in tilled plots and 8% in no-till, when compared to canola only rotation. When canola was grown with wheat and flax the disease incidence was reduced by 28% in tilled and 61% in no-till fields (Guo et al. 2005).

1.9.4 Tillage

Given that *L. maculans* relies on the decaying plant material to survive between growing seasons, crop residue is an important control point for black leg disease. When compared to conventional tillage, no-till and conservation tillage has the largest potential to increase disease risk due to changes in spore release patterns (McCredden et al. 2017; Krupinsky et al. 2002). When left on the surface unburied, infected crop residue releases more ascospores over time, making tillage an important control strategy to reduce inoculum (Krupinsky et al. 2002). The greatest viability of black leg inoculum is observed with 6-month-old stubble, decreasing dramatically after 18 months (Khangura et al. 2007). When leftover from a previous canola crop, 1-year old horizontal stubble released 75% more ascospores than stubble left standing vertically. Due to a decrease in decomposition when left standing, vertical stubble has advantage of potentially releasing spores a second year. The combination of early spore release from horizontal stems and gradual release from vertical stems causes inoculum to be released over long periods of time during wet years (McCredden et al. 2017).

When a Canadian study compared incidence of black leg between no-till and conventional tillage, disease incidence was lower in a no-till system over the course of 5 years in a barley-canola rotation (Kutcher and Malhi 2010). Although tillage still had a significant effect on reducing black leg incidence when a simple 2-year rotation (spring canola, spring wheat) was compared to continuous cultivation, it became less effective with diverse rotations. When no-till was observed with a diverse crop rotation (spring canola, spring wheat, flax, spring canola) incidence of blackleg was 33% lower than tilled plots (Guo and

Fernando 2005). Tillage helps reduce inoculum because of faster residue degradation, but having a strong rotation with a variety of crops also reduces disease pressure dramatically.

1.10 Host Resistance

Pathogenicity in *L. maculans* is based on pairs of complementary genes called avirulence (*Avr*) and resistance (*Rlm*). Host resistance to black leg relies on recognition between the pathogen's *Avr*-gene and the host's *Rlm* gene. When the host recognizes an *Avr*-gene with a corresponding *Rlm*-gene, cellular processes are triggered which lead to the activation of the hypersensitive response (Ansan-Melayah et al. 1998). When the pathogen expresses an *Avr*-gene that is unmatched by an *Rlm*-gene in the host, the plant is susceptible to disease (Hayward et al. 2012). To date, 16 *Avr* genes have been identified in *L. maculans*: *AvrLm1-2-3-4-5-6-7-8-9-10-11-S, AvrLepR1-LepR2-LepR3-LepR4* (Hayward et al. 2012; Van de Wouw et al. 2013; Balesdent et al. 2013; Yu et al. 2013).

Between monogenic and polygenic resistance, host-pathogen interactions differ greatly. Monogenic refers to single gene-for-gene interactions that confer resistance as early as the seedling stage as described above. The genes for monogenic resistance are usually dominant and can explain the majority of phenotypic variation for black leg resistance at the adult plant stage (Delourme et al. 2004; Raman et al. 2013). The term polygenic resistance refers to large amounts of genotypic variation that is associated with genes that contribute partial resistance (Delourme et al. 2004).

A major concern for using plants with only monogenic resistance is the selective pressure they put on pathogen populations. With short rotations and large populations of *L. maculans* interacting with a few *Rlm* genes, resistance can breakdown in as little as three

years with heavy selective pressure (Brun et al. 2000; Sprague et al. 2006). In Canada, many of the resistant canola cultivars included the resistance gene *Rlm3*. In Manitoba, a highly variable *L. maculans* population was analyzed for *Avr* genes. Within the population, the frequency of the corresponding *AvrLm3* was very low, indicating a breakdown in resistance (Zhang et al. 2016). Resistance 'breakdown' implies that the host resistance has not changed, but rather the pathogen population has been selected to exclude genes that allow recognition by the host. For example, if cultivars are grown with the gene *Rlm6*, there will be a shift toward isolates that lack the corresponding *AvrLm6* and a decrease in the effectiveness of *Rlm6*. During the next cropping year, if cultivars with *AvrLm9* are grown, then a different population of fungus will be selected for, limiting population boom each season. It is suggested that a rotation of cultivars with different resistance genes should minimize the build-up of isolates that are not recognized by a particular resistance gene (Marcroft et al. 2012). Polygenic resistance is much more appealing to farmers, as resistance breakdown occurs at a much slower rate due to less selective pressure on single genes.

1.11 Cost of Fitness

Fitness refers to the pathogen's ability to reproduce and carry on its lifecycle year to year. The concept of fitness helps explain how populations of pathogens are selected in a way that creates more virulent strains over time. Given that fitness can be measured in absolute or relative terms, several aspects are considered depending on the organism studied. For plant pathogens, fitness cost can be associated with reproductive rate, infection efficiency, or severity of disease (Pringle and Taylor 2002; Vera Cruz et al. 2000; Leach et al. 2001; Huang et al. 2006).

For *L. maculans*, infection efficiency and severity play a large role in determining isolate fitness. Isolates with many *Avr* genes are less likely to reproduce year to year due to broader range of hosts recognizing excess avirulence. Isolates with a narrow range of *Avr* genes are more likely to infect a host early on, causing more severe infections later in the season. An isolate with fewer *Avr* genes is harder to counter with resistant cultivars, due to a narrow range of *Avr* genes that can be harder to predict. (Vera Cruz et al. 2000; Huang et al. 2006; Pringle and Taylor 2002).

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CHAPTER 2: INVESTIGATING THE DISTRIBUTION AND DIVERSITY OF *LEPTOSPHAERIA MACULANS* IN NORTH IDAHO

2.1 Introduction

The inland northwest has a Mediterranean-like climate, with cool, wet winters and warm, dry summers. The average precipitation can range from 60 cm on the eastern edge (Washington-Idaho border) to less than 15 cm on the western edge (Columbia Basin). Common topography in the inland northwest is steep, with farmed slopes ranging from 30 to 45%. Steeper topography occurs in the intermediate to high precipitation zones where most of the annual cropping systems are found (Kok et al. 2009; USDA 1978).

In Idaho, 8,296 hectares of canola were harvested in 2016, contributing $6.63 million to the state economy at a state average price of $0.30/kg^{-1}. By comparison in 2016, Montana produced 24,000 hectares, Oregon produced 1,500 hectares, and Washington produced 12,500 hectares (USDA-NASS, 2013-2016).

Canola is susceptible to a number of diseases which can impact the host from seedling to maturity. In north Idaho, black leg (*Leptosphaeria maculans*), Rhizoctonia root rot (*Rhizoctonia solani*), and Sclerotinia white mold (*Sclerotinia sclerotiorum*) are the most concerning fungal pathogens. Other species of *Brassica* can succumb to similar diseases, including black leg, and act as alternate hosts in cropping systems. Incidence and severity of these diseases depend on farming practices, environmental conditions, and the intensity of crop rotations. (Bailey et al. 2003)

Black leg is attributed to the fungal pathogen *Leptosphaeria maculans* (anamorph *Phoma lingam*) which infects plants in the family Brassicaceae. This fungus can infect both

spring and winter cultivars of oilseed rape, and can be found in a wide range of climates, despite widely varying agricultural practices (West et al. 2001). Leaf lesions are the most common symptom of black leg seen in north Idaho. Lesions can be greyish white, pale brown, pale gray or gray green, round to irregular, and dotted with numerous black pycnidia (West et al. 2001; Bailey et al. 2003). Black leg gets its name from the lesions and dry sunken cankers that develop on the lower portion of the stem, which upon cutting open perpendicular to the stem will reveal a black discoloration. Only found in severe infections, cankers can girdle the plant, reducing yield and potentially lodging the host before ripening (West et al. 2001).

Black leg is considered a new world disease, with introduction scenarios suggesting that *L. maculans* originated in the United States and spread to many countries unknowingly (Dilmaghani et al. 2012). In Germany, the first formal description of black leg occurring on dried stems of red cabbage (*Brassica oleracea var. rubra*) was detailed by H. I. Tode as *Sphaeria lingam* in 1791, with the name later revised as *Phoma lingam* by J. Desmaiere in France in 1849. The first authentic report of black leg in the United States occurred in 1910 in Sandusky County, Ohio with a large distribution of infection in cabbage grown across the state. The first report of a black leg epidemic attributed to *P. lingam* (anamorph) was found in Onalaska, Wisconsin in 1911. The symptoms ranged from a loss of a few heads per field to nearly 75 percent loss, with disease spread across a radius of several miles. Symptoms in Wisconsin were described as stems with sunken lesions that extended about halfway through the stem. Lesions on the plants produced recognizable pycnidia relating to Tode's description in 1791(Henderson 1918).

Some of the first reports of black leg in Idaho were from farmers near Potlatch in the northern part of the state in 1990. However, there was no confirmation of black leg in these

earlier incidences (Jim Davis, personal communication). The presence of black leg in Idaho was confirmed following the observation of the disease in Boundary county near the Canadian border in 2011 (Agostini et al. 2013). In 2014, a grower producing industrial rapeseed in Lewiston, ID had canola samples intended for certified seed analyzed by Eurofins Scientific. The seed lot tested positive after a ten thousand seed sample was screened for black leg. Out of six trucks, half of those tested showed an incidence of up to 0.03% infected seed. Given that a 27 metric-ton truckload of canola would yield nearly 4.8 trillion seeds, a 0.02% average infection would result in nearly one million infected seeds (Jim Davis, personal communication). A preliminary survey was conducted in 2015, collecting samples in the Lewiston valley and Camas Prairie. Black leg was found in the majority of the samples collected, and 26 isolates were confirmed by PCR to be *L. maculans* (Fernando et al., unpublished).

Host resistance is conferred by a gene-for-gene interaction between the host plant and the pathogen. When a resistance gene (*Rlm*) in the plant and complementary avirulence gene (*Avr*) in the pathogen interact, a hypersensitive response is triggered (Ansan-Melayah et al. 1998). When the pathogen expresses an *Avr* gene that is unmatched by an *Rlm* gene in the host, the plant is susceptible to disease (Hayward et al. 2012). To understand how the pathogen infects surrounding crops, avirulence genes are studied to assist risk assessment and support efforts in plant breeding. To date, 16 *Avr* genes have been identified in *L. maculans: AvrLm1-2-3-4-5-6-7-8-9-10-11-S, AvrLepR1-LepR2-LepR3-LepR4* (Hayward et al. 2012; Van de Wouw et al. 2013; Balesdent et al. 2013). These genes can be screened by combining PCR assay and plant differentials with single genes for resistance.

The goals of this project were to determine how widespread the black leg epidemic was in north Idaho and to characterize this population to determine the presence and abundance of avirulence genes within the *L. maculans* population. The following study outlines the results of a survey conducted during 2016 and 2017 and the characterization of populations of *L. maculans* and *L. biglobosa*.

2.2 Materials and Methods

2.2.1 Survey and Sample Collection

A survey for black leg symptoms on canola crops was conducted in 2016 and 2017 by collecting samples from commercial farmers' fields. Fields were located across 50 individual sites in north Idaho spanning four counties. Scouting was conducted during June and November in 2016 as well as May and June in 2017. Each field was scouted for symptoms of black leg, and suspicious plant tissues were sampled for confirmation and isolate collection. Sampled material included leaves displaying typical black leg lesions, fresh stubble with gray lesions, and field residue displaying lesions with black pseudothecia. A maximum of 10 samples were collected at each location.

Collected samples consisted of multiple *Brassica* species, including winter canola, spring canola, industrial rapeseed, spring mustard, wild mustard, and oilseed crop residue from previous season (*B. napus, B. juncea, B. rapa, Sinapis alba,* and *S. arvensis*). Sample group was determined by the collection date, which is designated with a single letter. The first part of the isolate's name (A01) corresponds to the date and location the isolate was collected: A – 6/2/16, B –6/16/16, C – 11/16/16, D – 5/12/17, E – 5/25/17, F – 6/5/17, G – 6/6/17.

Sample locations included sites across Idaho, Latah, Lewis, and Nez Perce counties in Idaho as well as Spokane county in eastern Washington along the Idaho border (Table 2.1).

2.2.2 Fungal Isolation and Culture Maintenance

Leaf tissue showing symptoms of black leg was further dissected into 5 cm triangles encompassing individual lesions, then placed in petri plates with soaked germination paper. Samples were placed in the dark for at least 48 hr until pycnidiospores were produced. Once masses of pycnidiospores oozed from pycnidia, the ooze was streaked onto a water agar plate (18 g agar in 1 L distilled water) using forceps under a field microscope. Infected tissue that was isolated from stem or field residue were cut into 5 cm pieces encompassing lesion, soaked for 2 min in 1% bleach solution (8.25% NaClO), rinsed twice with sterile distilled water (SDW), and placed on water agar.

Plates were incubated in the dark at 15 to 20°C for at least 5 d. Hyphae were observed under a field microscope for growth, and individual hyphal tips were transferred onto PDA amended with antibiotic (PDA+; 1 L distilled water, 18 g agar, 24 g potato dextrose, 100 mg streptomycin, 100 mg tetracycline), grown and incubated as described above for 2 weeks before being placed in refrigerated storage at 4°C.

All isolates were maintained on PDA+ petri plates and stored at 4°C. Long-term storage was accomplished by dipping filter paper discs into a suspension of the fungus in potato dextrose broth (PDB) and allowing to dry for 4 hrs. Dry discs were stored in 1.5 ml centrifuge tubes at -20°C.

2.2.3 Inoculum Preparation and Pathogenicity Test

The identity of each isolate was confirmed by a pathogenicity test on a susceptible canola cultivar. A plug of agar medium was cut from the growing edge of an active mycelial culture, placed onto 20% V8 juice agar petri plates (V8; 800 ml distilled water, 18 g agar, 200 ml V8 vegetable juice, 2 g calcium carbonate), and maintained under plant lights (F40 T12 Plant and Aquarium, GE, USA) for 14 d at ambient room temperature (~21°C) (Ansan-Melayah et al. 1995). After incubation, each culture was flooded with 10 ml SDW, scraped with a microscope slide to dislodge spores, and filtered through Miracloth (Calbiochem, La Jolla, CA, USA) using a funnel in a 40 ml centrifuge tube (Liban et al. 2016). Inoculum was concentrated using an Eppendorf 5804 R centrifuge at 1500 rpm (16.1 RCF) for 10 min. Supernatant was poured off and remaining inoculum was suspended in 1 ml of SDW. Samples were transferred to 1.5 ml centrifuge tubes before storing at -20°C.

On the day of inoculation, frozen inoculum was added to SDW in microcentrifuge tubes. Concentration was adjusted to a desired range between 1.5-2.5x10^{7} spores/ml^{-1} using a hemocytometer. The canola cultivar 'Westar' was seeded into 72-cell seedling flats filled with Sun Gro potting medium (Sun Gro Horticulture, Agawam, MA, USA). Flats were spot watered daily and maintained in the greenhouse at 20 to 24°C with a 16 hr photoperiod. Host plants were grown for 7 to 10 d, and two seedlings were inoculated with a spore suspension of a single *L. maculans* isolate. Each lobe of the cotyledon was wounded using a pair of modified forceps, and a 10 uL droplet of prepared inoculum was pipetted onto each of the wounds of the cotyledon (two inoculated lobes per plant) and allowed to dry. Post inoculation, plants were transferred to tents and kept under high humidity for 48 to 60 hr (Van de Wouw et al. 2009). After removing trays from humidity tent, plants were watered with fertilizer using

20:20:20 (N:P:K). Additional fertilization using the same mixture took place after 7 d, post humidity tent. Plants were checked daily for emerging true leaves, which were removed to delay senescence of cotyledons (Liban et al. 2016).

Plants were evaluated 14 and 21 d after inoculation, with ratings based on the IMASCORE rating system (Figure 2.1). The IMASCORE scale comprises six infection classes (IC), where IC1 is the typical hypersensitive response (Figure 2.1A-C). IC2 represents a larger (1.5 to 3 mm) dark necrotic lesion (Figure 2.1D-F). IC3 is a nonsporulating lesion that may or may not show tissue collapse as in IC4 to IC6, but that is always sharply delimited by a dark necrotic margin that may extend within the lesion (Figure 2.1G-I). IC4 to IC6 are characterized by gray-green tissue collapse without a darkened margin, and showing no sporulation (IC4) (Figure 2.1J-L), a few pycnidia (IC5), or profuse sporulation (IC6) (Balesdent et al. 2001). IC1 is considered a strong resistance response (R), IC2 to IC3 are considered to be moderate resistance responses (MR), whereas IC4 to IC6 are considered to be susceptible (S)(Van de Wouw et al. 2009). The results were analyzed to confirm whether the isolate was pathogenic on canola and if the isolate was *L. maculans* or *L. biglobosa*. If an isolate was observed to be resistant, then it was subjected to another round of testing to confirm results.

2.2.4 DNA Extraction and Confirmation of Isolate Identity

Following pathogenicity tests, the identity of isolates was confirmed by sequencing of the ITS region of the rDNA. Mycelium was grown in a potato dextrose broth (PDB) (1 L distilled water, 24 g potato dextrose) by adding 10 uL of concentrated frozen inoculum to 15 mL of PDB. Cultures were grown in the dark for 7 d. Using forceps, mycelium was removed from the broth, dried on filter paper, then placed in lysing tubes. DNA was isolated from

mycelium using the FastDNA Kit (MP Biomedicals, Santa Ana, CA, USA) and BioSpec Mini Beadbeater (Biospec Products Inc., Bartlesville, OK, USA), following the protocol provided with the FastDNA Kit.

The PCR amplification of the ITS region was done as follows: 2 uL of template DNA was added to 15.9 ul SDW, 6 uL 5x Buffer, 10 mM dNTP, 10 pmol UN-UP18S42 F (5' CGTAACAAGGTTTCCGTAGGTGAAC), 10 pmol UN-LO28S576B R (5' GTTTCTTTTCCTCCGCTTATTAATATG), and 0.3 uL GoTaq (Promega, Sunnyvale, CA) (Bakkeren et al., 2000). A thermal cycler (5345 MasterCycler, Eppendorf, Hamburg, Germany) was used to amplify the DNA using the following protocol: 3 min at 94°C (1 cycle); 45 s at 92°C, 45 s at 60°C, and 60 s at 72°C (30 cycles); 10 min at 72°C; hold indefinitely at 15°C.

Cleaning of PCR product was completed using the Ultraclean PCR Cleanup Kit (Qiagen, Hilden, Germany). Prior to sequencing, samples were prepared as follows: 7.5 uL DSW, 5 uL of clean PCR product, 2.5 uL of 3.2 pmol primer were combined in a 1.5 ml centrifuge tube. Samples were sent to Elim Biopharm (Hayward, CA) for sequencing. Sequences were reviewed and edited using the software Genious (Auckland, New Zealand), then compared to published results (Mendes-Pereira et al. 2003). Sequence was analyzed by a BLAST search provided by NCBI (http://ncbi.nlm.nih.gov), under the following conditions: blastN suite, nucleotide collection (nr/nt) database, megablast (optimize for highly similar sequences) (Altschul et al. 1990). \

2.2.5 Characterization of Mating Types

Each PCR amplification of the MAT1/MAT2 region was done as follows: 2uL of template was added to: 10 uL SDW, 4 uL 5x Buffer, 10 mM dNTP, 10 pmol forward primer, 10 pmol reverse primer (Table 2), and 0.2 uL GoTaq. PCR reactions were carried out in an Eppendorf thermal cycler using the following protocol: 30 s at 94°C (1 cycle); 15 s at 55°C, 60 s at 72°C, and 60 s at 72°C (30 cycles); 5 min at 72°C, then held at 15°C (Zhongwei Zou, personal communication).

2.2.6 Characterization of Avirulence Genes by PCR

The presence of avirulence genes was determined by using specific primers for *AvrLm1-2-3-(4-7)-5-6-11*. For each reaction, 2 uL of template was added to: 10 uL SDW, 4 uL 5x Buffer, 10 mM dNTP, 10 pmol forward primer, 10 pmol reverse primer (Table 2.2), and 0.2 uL GoTaq. The DNA was amplified using an Eppendorf thermal cycler as follows: 30 s at 94°C (1 cycle); 30 s at 50°C, and 60 s at 72°C (30 Cycles); 5 min at 72°C, then held at 15°C. (Zhongwei Zou, personal communication).

To confirm a PCR reaction was successful, prepared samples were run through electrophoresis gels and compared to a ladder. The electrophoresis gel was prepared with 1% agarose and ethidium bromide (10 mg/mL). Samples were compared to a ladder (1kb+, Thermo Fisher Scientific, USA) with negative and positive controls.

2.2.7 Characterization of Avirulence Genes by Host Differentials

Each isolate of *L. maculans* and *L. biglobosa* was inoculated onto a series of host differentials to further characterize the presence of avirulence genes (Table 2.3). Host differentials consisted of a set of *B. napus* lines or cultivars and *B. juncea* cultivar Cutlass.

Each carries a specific *Rlm* gene(s) to identify *Avr* alleles in *L. maculans* and *L. biglobosa* isolates. The set of differentials consisted of: Westar (no *Rlm*; Delourme et al. 2004), Columbus (*Rlm1,3*; Balesdent et al. 2006), Glacier (*Rlm2,3;* Balesdent et al. 2005), Bristol (*Rlm2,9*; Balesdent et al. 2006), 02.22.2.1 (*Rlm3;* Gout et al. 2006), Jet Neuf (*Rlm4;* Balesdent et al. 2005), Cutlass (*Rlm5,6*; Liban et al. 2016), 01.23.2.1 (*Rlm7;* Dilmaghani et al. 2009), Goeland (*Rlm9;* Balesdent et al. 2006), Topas LepR1 (*RlmLepR1;* Larkan et al. 2016), Topas LepR2 (*RlmLepR2*; Larkan et al. 2016), and Topas LepR3 (*RlmLepR3;* Larkan et al. 2016) (Table 2.3). Plants were grown, inoculated, and scored using the IMASCORE system as described above. For the differential, six plants were grown for each screening cultivar and four inoculation sites were measured per plant, for a total of 24 lesion ratings per differential line.

Based on this differential set, the corresponding *Avr* genes were deduced from *L. maculans* and *L. biglobosa* isolates: *AvrLm1,2,3,4,7,9, AvrLepR1*, *AvrLepR2*, and *AvrLepR3*. The genes *AvrLm5,6,8,10, 11,S,* and *AvrLepR4* could not be individually assessed via greenhouse differential due to a lack of obtained cultivars carrying corresponding resistance genes.

Table 2.1: List of sampled locations which produced isolates.

The sample group corresponds to the date the isolate was collected: A – 6/2/16, B –6/16/16, C – 11/16/16, D – 5/12/17, E – 5/25/17, F – 6/5/17, G – 6/6/17. The location number was assigned to samples in the order they were collected on the date. The species category designates the crop name and whether the crop was a spring (S) or winter (W) variety. Abbreviated counties in the table are Nezperce, ID (NPERCE) and Spokane, WA (SPOKN).

Sample* group	Location	Coordinates	Date	Sampled Crop**	County***	Nearest Town
A	1	46.347778, -116.854737	6/2/2016	W. Rapeseed	NPERCE	Lewiston, ID
A	2	46.322145, -116.865498	6/2/2016	W. Rapeseed	NPERCE	Lapwai, ID
A	4	46.230585, -116.566878	6/2/2016	W. Canola	LEWIS	Winchester, ID
A	5	46.230585, -116.566878	6/2/2016	S. Canola	LEWIS	Winchester, ID
A	7	46.286920, -116.360700	6/2/2016	W. Canola	IDAHO	Ferdinand, ID
A	8	45.964973, -116.264872	6/2/2016	S. Canola	IDAHO	Fenn, ID
A	9	45.997874, -116.211100	6/2/2016	W. Canola	IDAHO	Denver, ID
A	10	45.997624, -116.138650	6/2/2016	S. Canola	IDAHO	Denver, ID
A	11	45.961382, -116.139175	6/2/2016	W. Canola	IDAHO	Grangeville, ID
A	12	46.219258, -116.264130	6/2/2016	W. Canola	IDAHO	Nezperce, ID
A	12b	46.219258, -116.264130	6/2/2016	Wild Mustard	IDAHO	Nezperce, ID
B	2	46.600218, -116.562626	6/16/2016	W. Canola	NPERCE	Leland, ID
B	3	46.573951, -116.436457	6/16/2016	W. Canola	NPERCE	Cavendish, ID
B	4	46.579528, -116.811950	6/16/2016	S. Mustard	LATAH	Genesee, ID
B	5	46.579528, -116.811950	6/16/2016	S. Canola	LATAH	Genesee, ID
C	1	46.347933, -116.853243	11/16/2016	W. Rapeseed	NPERCE	Lewiston, ID
C	2	46.237486, -116.506874	11/16/2016	S. Canola	LEWIS	Craigmont, ID
C	3	46.248889, -116.431389	11/16/2016	W. Canola	LEWIS	Craigmont, ID
C	4	45.953889, -116.243611	11/16/2016	W. Canola	IDAHO	Fenn, ID
D	1	46.724623, -116.960155	5/12/2017	W. Canola	LATAH	Moscow, ID

D	2	46.724891, -116.950113	5/12/2017	W. Canola	LATAH	Moscow, ID
D	3	45.953889, -116.243611	5/12/2017	W. Canola	IDAHO	Fenn, ID
D	4	45.961111, -116.099167	5/12/2017	W. Canola	IDAHO	Grangeville, ID
D	5	46.004722, -116.097778	5/12/2017	W. Canola	IDAHO	Grangeville, ID
D	6	46.047778, -116.181111	5/12/2017	W. Canola	IDAHO	Denver, ID
D	7	46.010000, -116.180556	5/12/2017	W. Canola	IDAHO	Denver, ID
D	8	46.012500, -116.201389	5/12/2017	W. Canola	IDAHO	Denver, ID
D	10	46.248889, -116.431389	5/12/2017	W. Canola	LEWIS	Craigmont, ID
D	12	46.579908, -116.945993	5/12/2017	W. Canola	LATAH	Genesee, ID
E	2	47.382147, -117.078690	5/25/2017	W. Canola	SPOKN	Fairfield, WA
F	1	46.579908, -116.945993	6/5/2017	W. Canola	LATAH	Genesee, ID
F	2	46.585246, -116.950703	6/5/2017	W. Canola	LATAH	Genesee, ID
G	1	46.357272, -116.768457	6/6/2017	W. Canola	NPERCE	Lapwai, ID
G	2	46.366123, -116.739221	6/6/2017	W. Canola	NPERCE	Lapwai, ID
G	3	46.384304, -116.649165	6/6/2017	W. Canola	NPERCE	Culdesac, ID
G	4	46.384223, -116.635861	6/6/2017	W. Canola	NPERCE	Culdesac, ID
G	5	46.577046, -116.562376	6/6/2017	W. Canola	LATAH	Kendrick, ID
G	6	46.705249, -116.792118	6/6/2017	S. Canola	LATAH	Troy, ID

* A – 6/2/16, B –6/16/16, C – 11/16/16, D – 5/12/17, E – 5/25/17, F – 6/5/17, G – 6/6/17
** W. – Winter variety, S. – Spring variety
*** NPERCE- Nez Perce County, ID; SPOKN- Spokane County, WA

Table 2.2: List of primers used for PCR differential and mating type testing.

Primer Name	Sequence (5' to 3')	Reference
AvrLm1F	CTATTTAGGCTAAGCGTATTCATAAG	Gout, 2006
AvrLm1R	GCGCTGTAGGCTTCATTGTAC	Gout, 2006
AvrLm2F	CGTCATCAATGCGTTCGG	Ghanbarnia, 2014
AvrLm2R	CTGGATCGTTTGCATGGA	Ghanbarnia, 2014
AvrLm3ext-SpelF	GAGAGAACTAGTCTGTTAAATGCCTGCTGT	Plissonneau, 2016
AvrLm3ext-XholR	GAGAGACTCGAGCGCGCTTATGTTAGAATC	Plissonneau, 2016
AvrLm4-7 ext - Lo	GATGGATCAACCGCTAACAA	Parlange, 2009
AvrLm4-7 ext - Up	TATCGCATACCAAACATTAGGC	Parlange, 2009
AvrLm6F	TCAATTTGTCTGTTCAAGTTATGGA	Fudal, 2007
AvrLm6R	CCAGTTTTGAACCGTAGTGGTAGCA	Fudal, 2007
AvrLmJ1F	ACAACCACTCTTCTTCACAGT	Van de Wouw, 2013
AvrLmJ1R	TGGTTTGGGTAAAGTTGTCCT	Van de Wouw, 2013
AvrLm11-L (uP119060_L)	CAAGTTGGATCTTTCTCATTCG	Balesdent, 2013
AvrLm11-U2 (uP119060_U)	TGCGTTTCTTGCTTCCTATATTT	Balesdent, 2013
MAT-1.1	CTCGATGCAATGTACTTGG	Cozijinsen, 2003
MAT-1.2	AGCCGGCGGTGAAGTTGAAGCCG	Cozijinsen, 2003
MAT-R	TGGCGAATTAAGGGATTGCTG	Cozijinsen, 2003

Table 2.3: List of cultivars used in greenhouse differential

Cultivars	Type	Rlm genes	Source*	Reference
Westar	S. Canola	None	UIDAHO	Delourme et al. 2004
Columbus	W. Canola	1, 3	INRA	Balesdent et al. 2006
Glacier	W. Canola	2, 3	NCRPIS	Balesdent et al. 2005
Bristol	W. Canola	2, 9	INRA	Balesdent et al. 2006
02.22.2.1	W. Canola	3	INRA	Balesdent et al. 2006
Jet Neuf	W. Canola	4	UIDAHO	Balesdent et al. 2005
Cutlass	S. Mustard	5,6	UIDAHO	Liban et al. 2016
01.23.2.1	W. Canola	7	INRA	Dilmaghani et al. 2009
Goeland	W. Canola	9	INRA	Balesdent et al. 2006
Topas LepR1	S. Canola	LepR1	AAFC	Larken et al. 2016
Topas LepR2	S. Canola	LepR2	AAFC	Larken et al. 2016
Topas LepR3	S. Canola	LepR3	AAFC	Larken et al. 2016
*AAFC- Agriculture and Agri-food Canada, INRA- French National Institute for Agricultural Research, UIDAHO- University of Idaho Canola Breeding Program, NCRPIS- United States Department of Agriculture - North Central Regional Plant Introduction Station.				

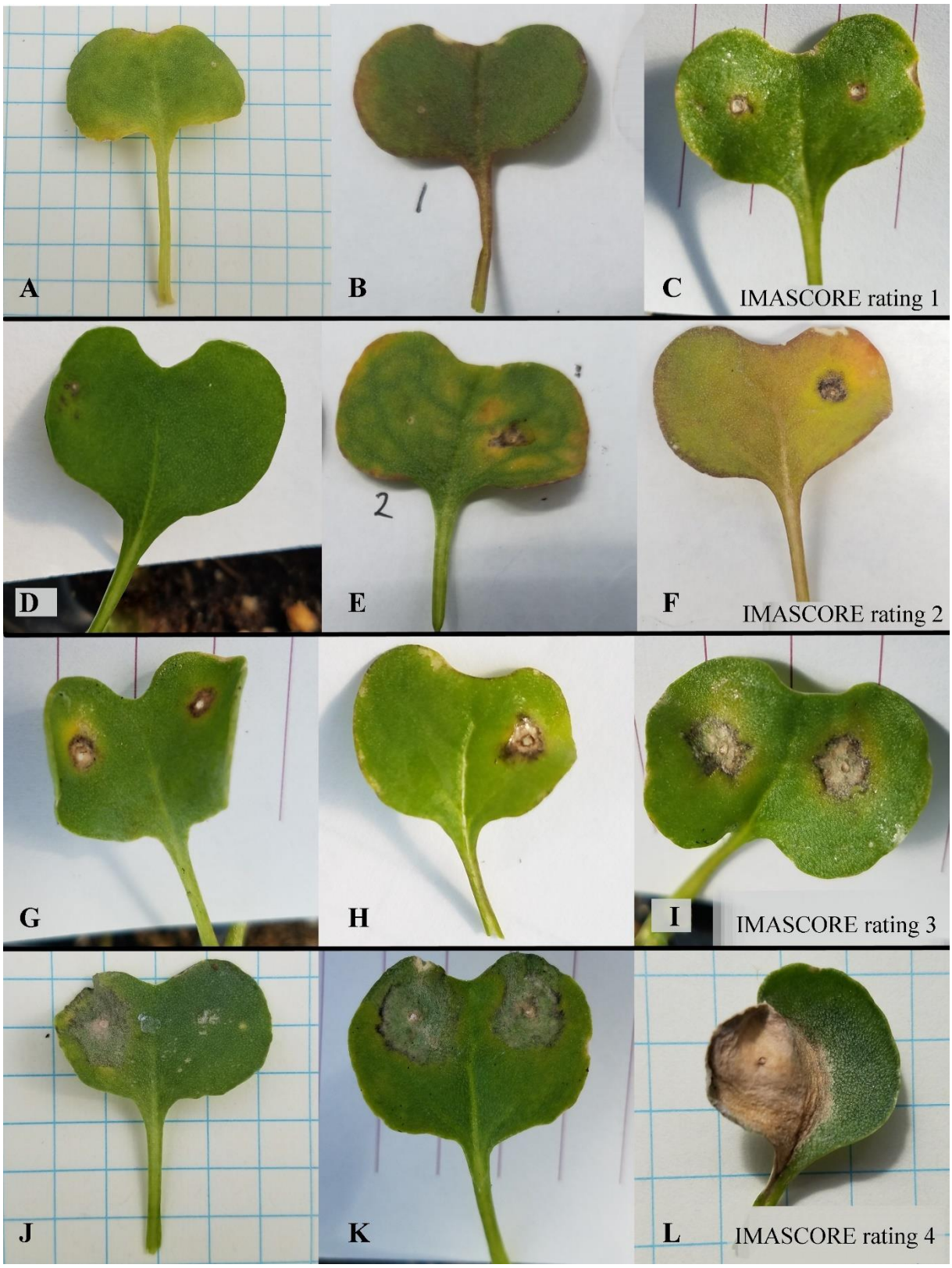


Figure 2.1: IMASCORE rating system

2.3 Results

2.3.1 Survey of North Idaho

Affected leaves of *Brassica* species in north Idaho were collected, along with suspicious stems and field residue (Table 2.1). A total of 50 unique sites were examined for the presence of black leg in north Idaho. Of these, black leg was confirmed in 39 (78%) of the sites following the identification and confirmation of *L. maculans* and *L. biglobosa* using pathogenicity tests and PCR amplification of the ITS region of the rDNA. There are 72 isolates from 2016 and 65 isolates from 2017.

In total, 125 isolates of *L. maculans* and 10 *L. biglobosa* were collected from north Idaho counties: Idaho county (72, 4), Latah (12, 4), Lewis (19, 0), Nezperce (22, 2). Additionally, two *L. maculans* isolates were obtained from samples collected in Spokane county, Washington near the Idaho border (Figure 2.2).

2.3.2 Identification of Mating-type Alleles in L. maculans

Mating types were identified using PCR, with each isolate being evaluated for both *MAT1* and *MAT2*. The survey of 127 *L. maculans* isolates resulted in 70 isolates with *MAT1* (55%) and 57 isolates with *MAT2* (45%) (Figure 2.3A). Subpopulations are categorized by county, based on the location that samples were collected. Both mating types were found in every county in nearly equal proportions (Figure 2.3B-E).

2.3.3 Characterization of Avirulence Genes by PCR

Using PCR techniques, seven alleles known to confer avirulence were screened: *AvrLm1, AvrLm2, AvrLm3, AvrLm4-7, AvrLm5, AvrLm6, AvrLm11* (Table 2.2). Of the 137 isolates of *L. maculans* and *L. biglobosa* tested, seven *L. biglobosa* isolates tested negative for all mentioned alleles. Of the 127 *L. maculans* isolates, the avirulence genes were found in the following frequencies: *AvrLm1* (0.7%), *AvrLm2* (0%), *AvrLm3* (17%), *AvrLm4-7* (91%),

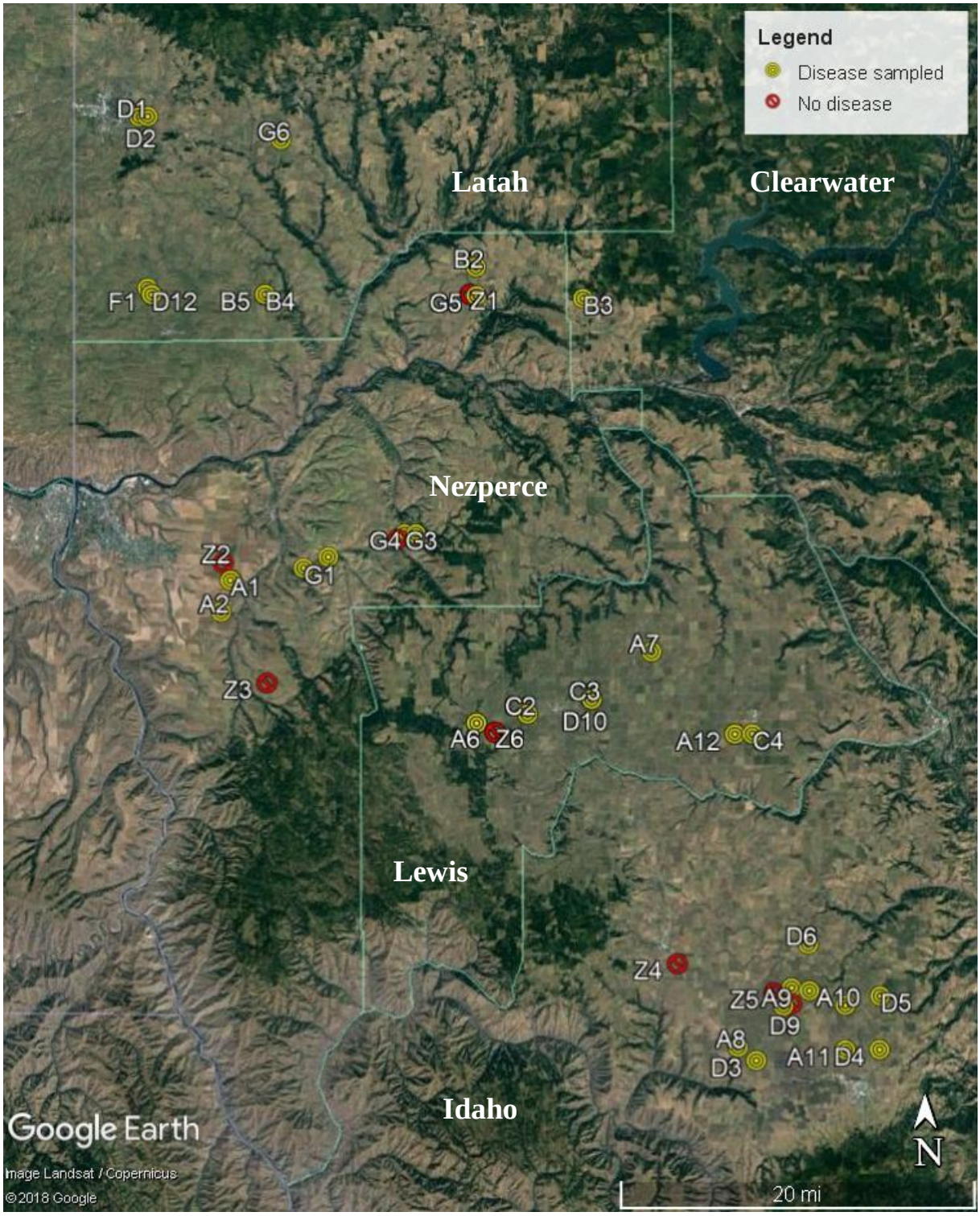


Figure 2.2: Map of black leg sampling locations in north Idaho. Data points on map correspond to locations found in Table 1. Some locations overlap so not all individual fields are displayed.

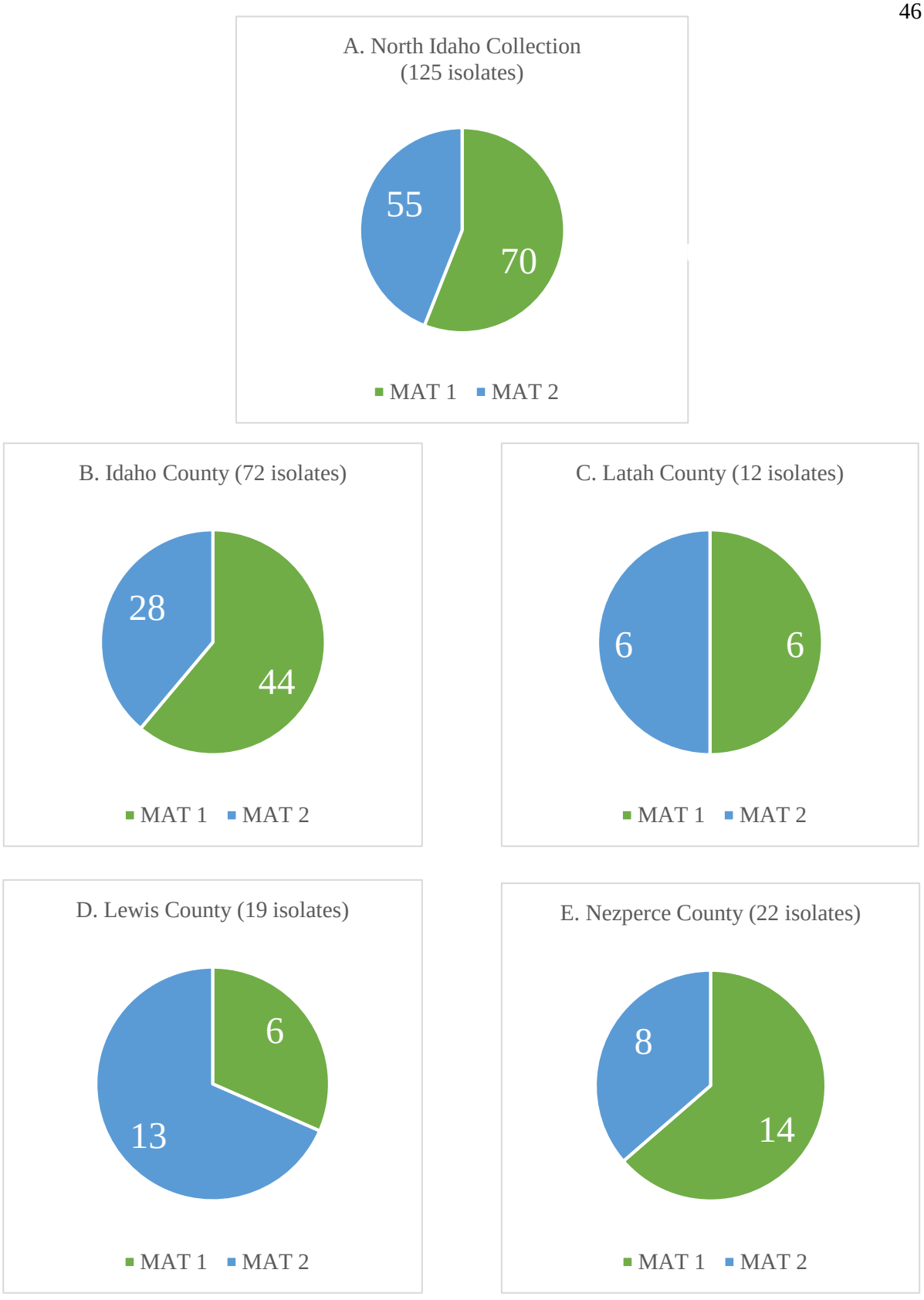


Figure 2.3: Frequency of *L. maculans* mating types in north Idaho population and subpopulations.

AvrLm5 (91%), *AvrLm6* (91%), *AvrLm11* (97%). Within the ten *L. biglobosa* isolates, two tested positive for *AvrLm5* and *AvrLm6,* and one isolate tested positive for *AvrLm4-7*.

2.3.4 Characterization of Avirulence Genes by Plant Differentials

A collection of plant differential lines of *B. napus* and *B. juncea* were inoculated with each isolate in the collection. Based on greenhouse differentials, the corresponding avirulence genes were deduced from *L. maculans* and *L. biglobosa* isolates: *AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm7, AvrLm9, AvrLepR1, AvrLepR2,* and *AvrLepR3.* Of the 137 isolates tested on the differentials, ten showed a resistant response on all cultivars, confirming them as *L. biglobosa*. Out of the 127 *L. maculans* isolates, the avirulence genes were found in the following frequencies: *AvrLm1* (4%), *AvrLm2* (3%), *AvrLm3* (0%), *AvrLm4* (7%), *AvrLm7* (100%), *AvrLm9* (3%), *AvrLepR1* (91%), *AvrLepR2* (69%), and *AvrLepR3* (34%).

2.3.5 Avirulent Alleles in Isolate Collection and Race Structure in Idaho

Based on a combination of PCR and greenhouse differentials, the corresponding avirulence genes were deduced from *L. maculans* isolates: *AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm9, AvrLm11, AvrLepR1, AvrLepR2,* and *AvrLepR3*. Of the 127 *L. maculans* isolates, the avirulence genes were found in the following frequencies (Figure 4): *AvrLm1* (4%), *AvrLm2* (3%), *AvrLm3* (15%), *AvrLm4* (7%), *AvrLm5* (94%), *AvrLm6* (94%), *AvrLm(7)* (100%), *AvrLm9* (2%), *AvrLm11* (98%), *AvrLepR1* (94%), *AvrLepR2* (69%), *AvrLepR3* (40%).

A similar distribution of *Avr* genes was found in each of the four counties surveyed (Figure 5). *AvrLm2* was found only in Idaho county, *AvrLm4* was found in Idaho and Nezperce counties and *AvrLm9* was only found in Idaho county. *AvrLm5, AvrLm6, AvrLm7, AvrLm11* and *AvrLepR1* were the most common *Avr* genes within each county, being found in

at least 67% of the population within each county. Avirulence genes found in the four counties were examined using chi-square analyses. When the distribution of the most popular genes was examined, there was no significant difference between distributions of avirulence genes across the sampled counties ($\chi^2_{21\ df} = 8.1$, p=0.9947). The majority of the difference between counties is explained by the low frequencies of the following avirulence genes: *AvrLm 1, 2, 3, 4, 9*.

The avirulence genes identified in each isolate, gathered from both PCR and plant differentials, were combined to determine a race for each isolate. There were 38 unique combinations of genes in the isolate collection, but only six races were found to contain five or more isolates (Table 4). The three most common races that make up 53% of the collection are: *5-6-7-11-LepR1-LepR2* (19%), *5-6-7-11-LepR1-LepR2-LepR3* (18%), and *5-6-7-11-LepR1* (16%) (Figure 6). By county, the most common races were *5-6-7-11-LepR1-LepR2-LepR3* (25%, Idaho), *5-6-7-11* (25%, Latah), *3-5-6-7-11-LepR1-LepR2* and *5-6-7-11-LepR1-LepR2* (each 21%, Lewis), and 5-6-7-11-LepR1 and *5-6-7-11-LepR1-LepR2* (each 23% Nezperce). Genetic races found in the four counties were examined using chi-square analyses. When black leg races were examined across different counties there was a significant interaction between race and location ($\chi^2_{18\ df} = 40.0$, p=0.002). This test was biased, as not every race in the chi square test was found in every county and the sample size outside of Idaho county was noticeably smaller. The most popular races found in north Idaho are all small variations of avirulence gene combinations. Given a small sample size, these variations did not have enough observations to amount to an unbiased test, with multiple values amounting to zero. This does not change the bottom line, being that different races were found in different locations.

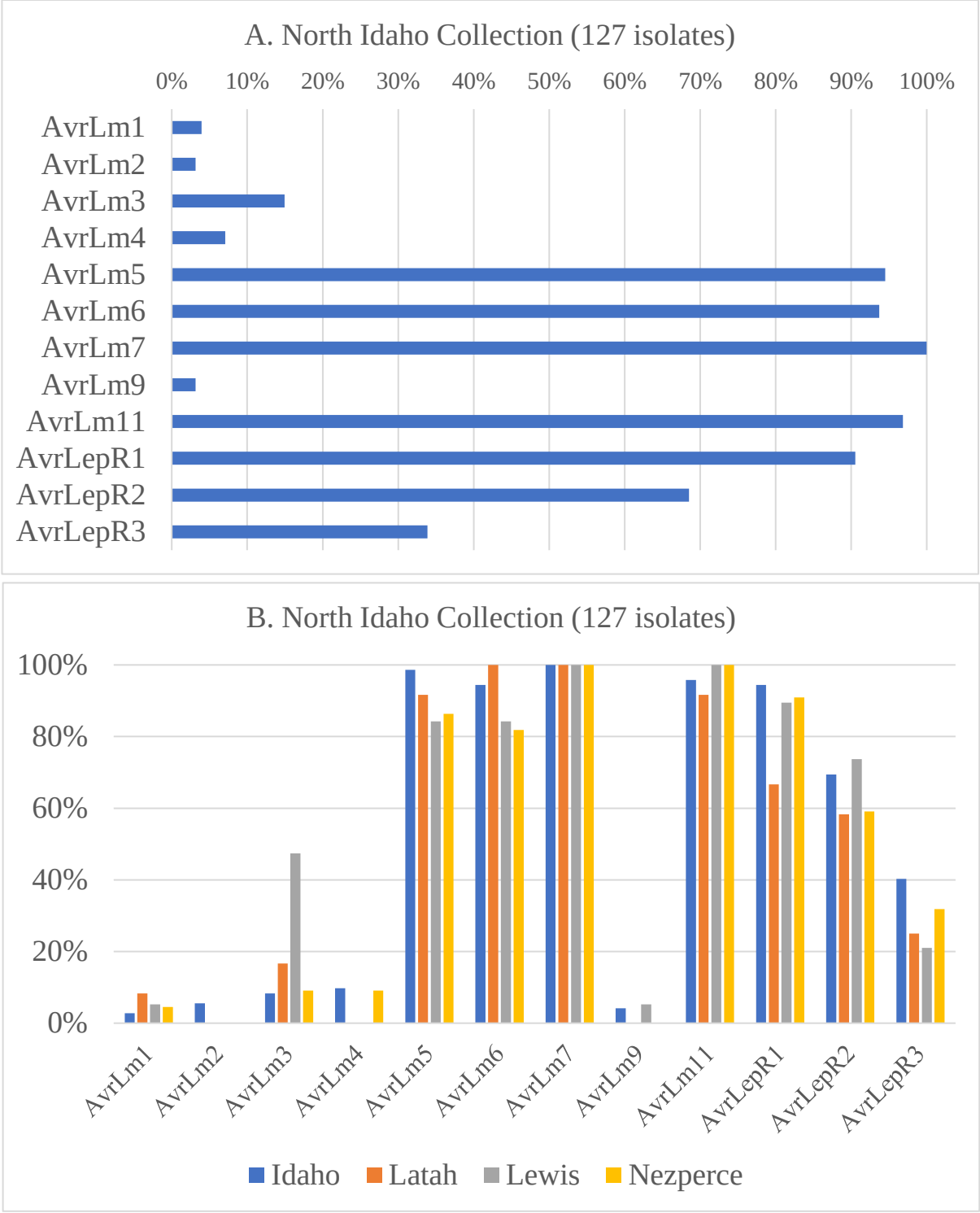


Figure 2.4: (A.) Percentage of isolates in the north Idaho collection containing specific avirulence genes based on PCR and plant differentials. (B.) Percentage of isolates in the north Idaho collection containing specific avirulence genes, split by representative county.

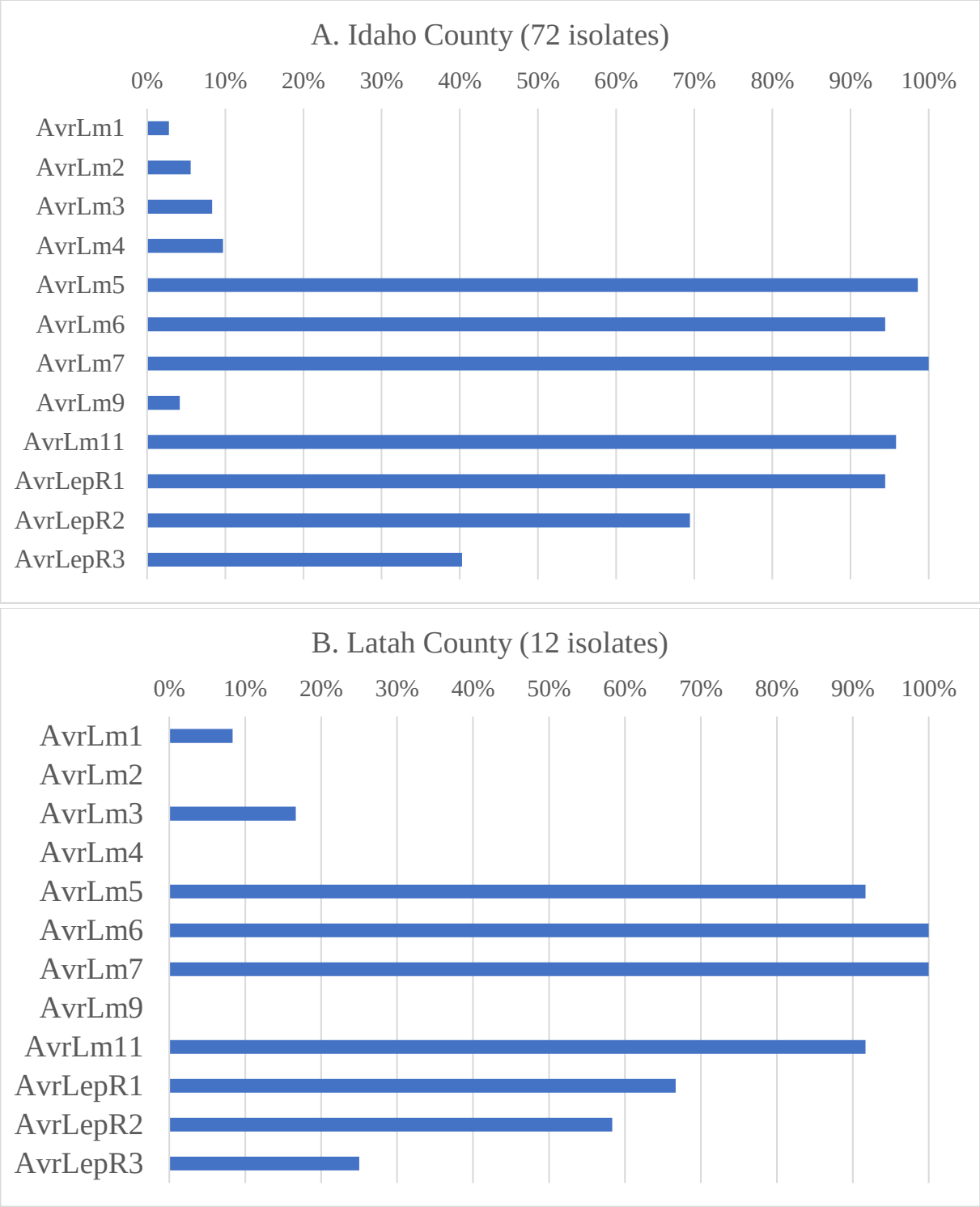
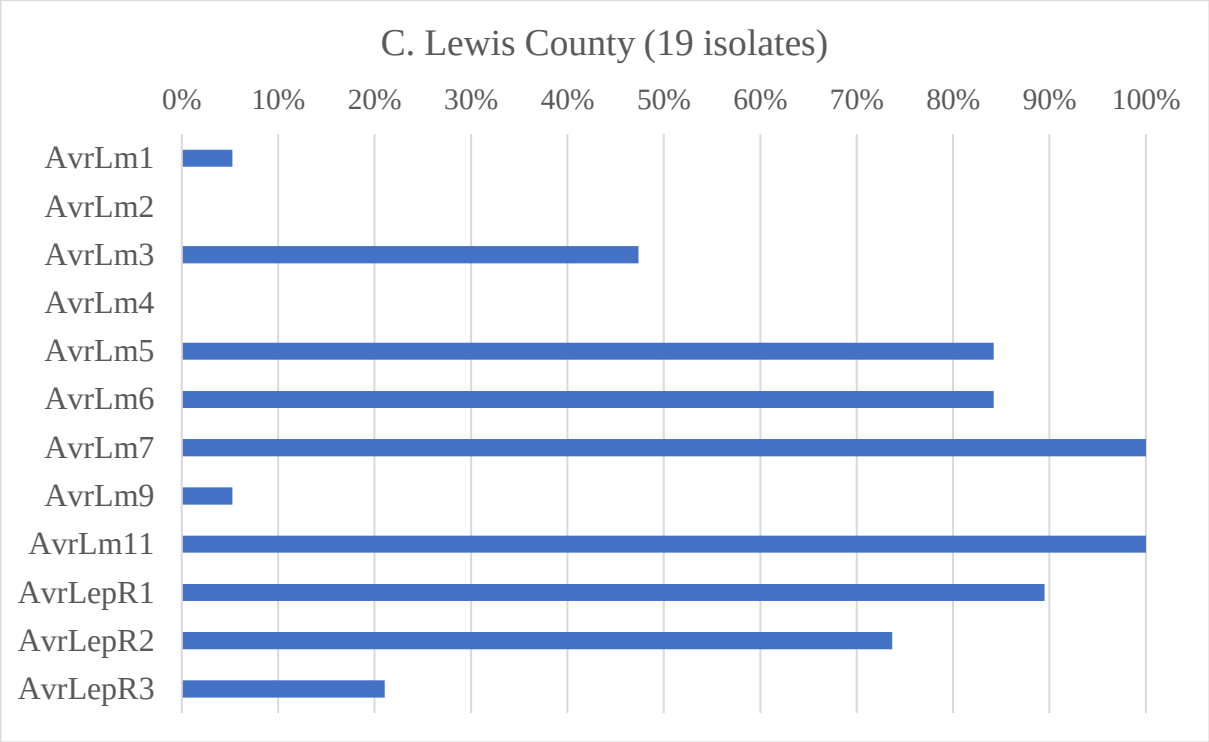


Figure 2.5 : Percentage of isolates in north Idaho collection subpopulations containing specific avirulence genes based on PCR and plant differentials.



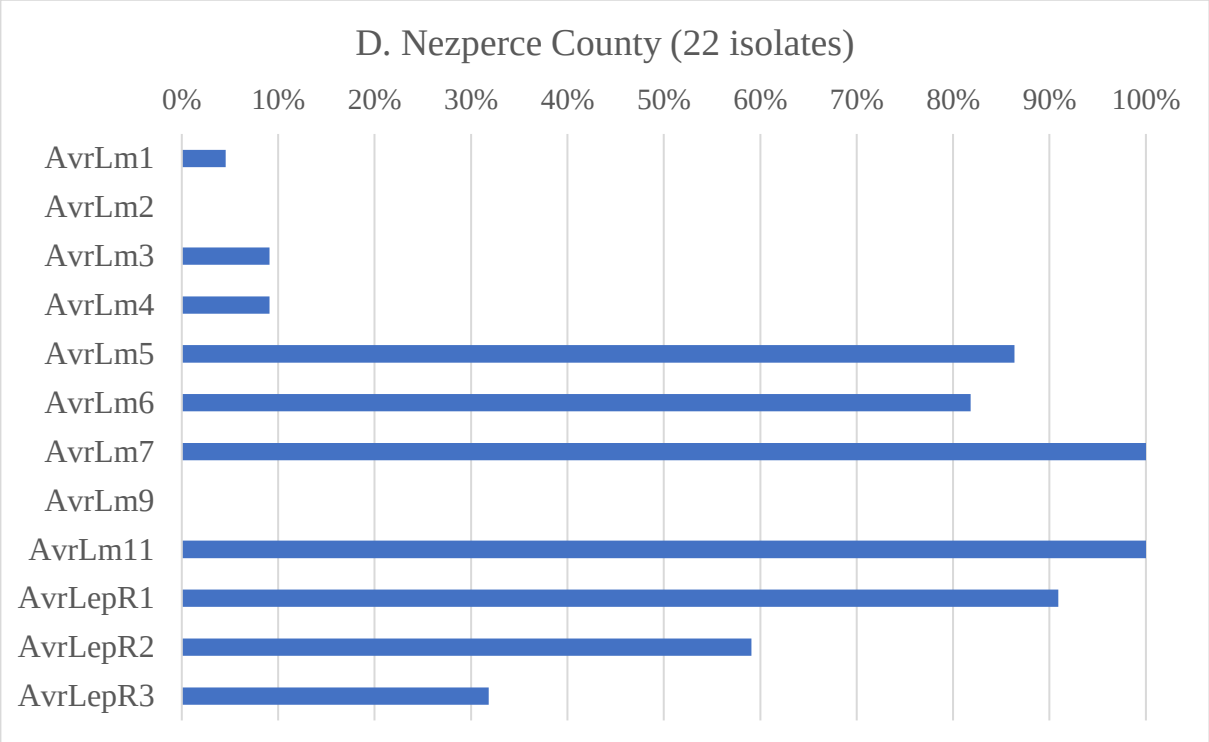


Figure 2.5 cont.: Percentage of isolates in north Idaho collection subpopulations containing specific avirulence genes based on PCR and plant differentials.

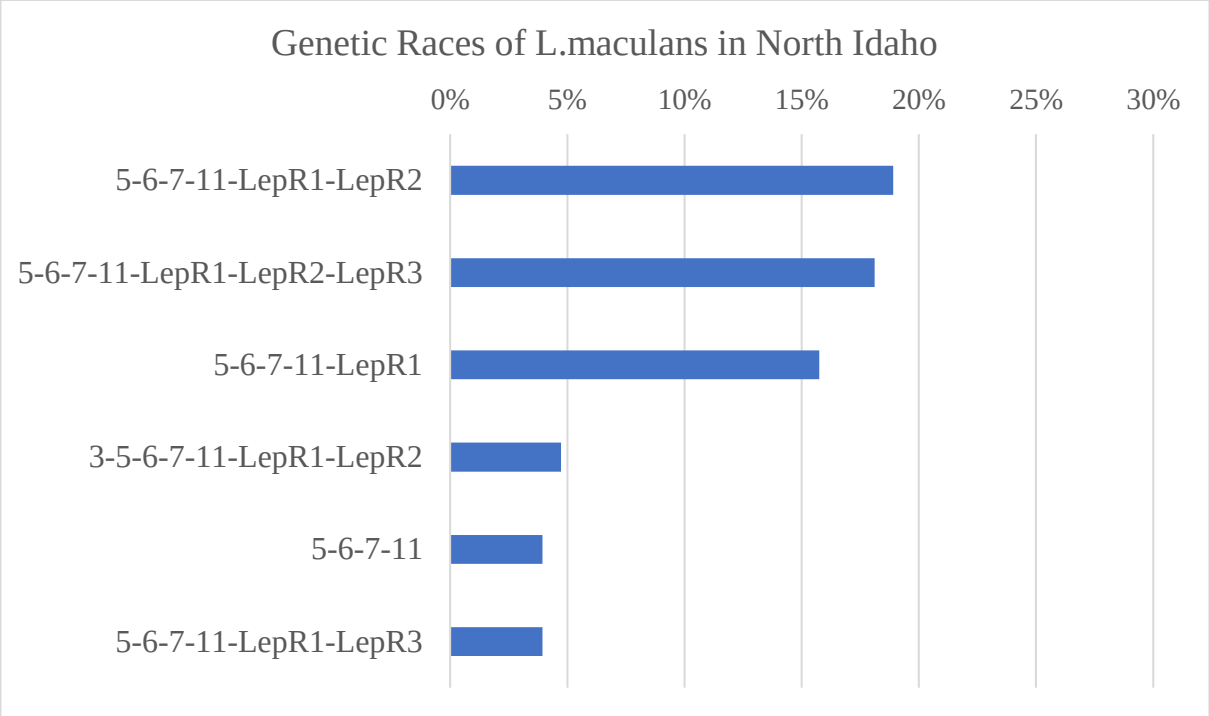


Figure 2.6: Percentage of isolates across the north Idaho Collection that belong to a specific genetic race. Only races identified in five or more isolates are listed.

Table 2.4: Genetic races of *L. maculans* in north Idaho collection.

Avirulence Genes Present	#Isolates
5-6-7-11-LepR1-LepR2	24
5-6-7-11-LepR1-LepR2-LepR3	23
5-6-7-11-LepR1	20
3-5-6-7-11-LepR1-LepR2	6
5-6-7-11	5
5-6-7-11-LepR1-LepR3	5
3-5-6-7-11-LepR1-LepR2-LepR3	4
3-5-6-7-11-LepR1	3
1-3-4-6-7-11-LepR1-LepR2	2
5-6-7-11-LepR2	2
5-6-7-9-11-LepR1-LepR2	2
5-7-11-LepR1	2
5-7-11-LepR1-LepR2	2
5-7-11-LepR1-LepR2	2
5-7-11-LepR1-LepR2-LepR3	2
5-7-LepR1-LepR2-LepR3	2
6-7-11.	2
6-7-11-LepR1-LepR2	2
1-4-5-6-7-9-11	1
1-5-6-7-11-LepR1-LepR2	1
1-6-7-LepR1-LepR2	1
2-3-5-6-7-11-LepR1-LepR2-LepR3	1
2-5-6-7-11-LepR1-LepR2-LepR3	1
2-5-6-7-11-LepR1-LepR3	1
2-5-6-7-LepR1-LepR2	1
3-4-5-6-7-11-LepR1-LepR2	1
3-5-6-7-11-LepR1-LepR3	1
3-5-6-7-9-11-LepR1-LepR2-LepR3	1
3-5-6-7-LepR1-LepR2	1
4-5-6-7-11-LepR1	1
4-5-6-7-11-LepR1-LepR2	1
4-5-6-7-9-11-LepR1-LepR2-LepR3	1
4-5-7-11-LepR1-LepR2	1
4-6-7-11	1
6-7-11-LepR1-LepR2-LepR3	1
6-7-11-LepR2	1
7-11-LepR1	1
7-11-LepR1-LepR2	1

2.4 Discussion

The goals of this project were to determine how widespread black leg was across north Idaho counties and to characterize this population to determine the presence and abundance of avirulence genes. This is the first comprehensive survey conducted in north Idaho to examine the extent of canola fields infected with black leg. Previous samples have been collected and confirmed for black leg, but only give a small glimpse of the overall situation. *Leptosphaeria maculans* was found widespread throughout the primary canola production region of northern Idaho. Upon examining a collection of isolates several races were identified, with the most common avirulence genes in the collection being: *AvrLm5*, *6*, *7*, *11*, *AvrLepR1*, and *AvrLepR2*.

The Camas Prairie is a large canola producing region that extends within the borders of Idaho and Lewis county. Claiming responsibility for most of north Idaho's canola acreage, Idaho county consequently represents half of the collection. During the collection of samples within the county, black leg was found in nearly every field of canola sampled. When sampling was done across other counties, canola fields were less frequent possibly due to climate and rotation popularity.

The majority of isolates were sampled from winter canola, while 20% of isolates were sampled from spring canola. Most of the sampling took place in June which may have led to a higher number of winter crops sampled. However, in the sampled region of Idaho county, winter canola is grown more often than spring due to climate, which also affected sample rates. Studies completed in western Canada often sample stems and stem lesions from spring oilseed, due to a limiting climate that favors spring crops (Hwang et al. 2016; West et al. 2001).

Winter canola will often show more developed symptoms when initial infection occurs around the seedling stage in the fall (West et al. 2001). No severe symptoms, including base stem cankers, were observed during the survey. Leaf lesions were sampled most frequently during collection. Western Canada and other regions with more severe disease symptoms often sample stems and stem lesions. In these cases, severity is assessed by taking a cross sections of the lower stem, scoring the progression of infected tissue to determine the disease impact (Hwang et al. 2016).

For sexual reproduction to occur and ascospores to be produced, isolates of *L. maculans* with MAT1 and MAT2 mating types must be present (Cozijnsen and Howlett 2003). While pseudothecia of *L. maculans* were not directly observed during field surveys, the nearly equal proportion of MAT1 (55%) and MAT2 (45%) suggest that sexual recombination is occurring in Idaho. Within counties, there was an equal proportion of both mating types in Latah county, while the largest skewed subpopulation was Lewis county (32% MAT1, 68% MAT2), although many of the isolates from Lewis county were taken from the same field. Given that both mating types are in the region, *L. maculans* is likely producing pseudothecia that serve as survival structures and release ascospores as inoculum every year to start new epidemics. The timing of spore release is currently unknown for north Idaho and currently does not occur in a way that causes severe symptoms on plants.

The occurrence of both mating types within a population is important in the lifecycle of *L. maculans* since sexual recombination introduces greater genetic variation (Cozijnsen and Howlett 2003). When selection pressure is applied, variability increases across the disturbed ecological habits. Common control factors like fungicides can select for populations resistant to certain modes-of-action, rendering common chemicals less effective over time (Hwang et

al. 2016). When crops with the same genetic resistance are grown consistently, certain avirulence genes may reduce in frequency within a population in as little as three years (Marcroft et al. 2012; Brun et al. 2010). Integrated pest management systems that rotate management strategies help reduce selection pressure that forces *L. maculans* to adapt and overcome.

Canola in north Idaho is grown in a Mediterranean-like climate, with cold wet winters and dry hot summers (Kok et al. 2009). The pathogen is successful in dry Mediterranean climates which generally have mild winter temperatures and hot summers near the sea or severely cold temperatures in the winter with hot dry summers inland (Kassam et al. 2012; West et al. 2001). Survival of the disease is affected by wetness and soil temperature, which control the rate of residue degradation (Khangura et al. 2007).

Rate of residue degradation decreases in environments with dry summers and cold winters. In the dry Western Australia, residue can remain an inoculum source for up to 4 years. While in the wetter south east region, oilseed rape residues declined in volume by 90% in 1 year, dramatically reducing ascospore production the following season (West et al. 2001). Resulting from a perfect combination of environmental factors, Western Australia faced losses approaching $50 million in 1999 (Khangura et al. 2007). This occurred when producers planted oilseed in the wet season in which rainfall triggered ascospore production. This increase of inoculum during seedling establishment resulted in early infections, leading to plant death or severe cankers later in the growing season, dramatically reducing yield (West et al. 2001).

PCR techniques allowed the for the screening of avirulence genes for which there are not currently plant differentials and include: *AvrLm(4-7), AvrLm5, AvrLm6* and *AvrLm11*. The

presence of *AvrLm(4-7)* was detected in 91% of the population alongside *AvrLm5* and *AvrLm6*. The PCR screening found more isolates in the collection with *AvrLm3* (17%) than greenhouse differentials, which found none. Given the expression of *AvrLm4-7* blocks *AvrLm3* and *AvrLm9,* the nonexistence of *AvrLm3* in the greenhouse differentials was expected (Ghanbarnia et al. 2018; Plissonneau et al. 2016). In this study, 100% of the isolates contained *AvrLm7,* effectively blocking the expression of *AvrLm3*.

Compared to PCR screening, the greenhouse differential resulted in a higher frequency of *AvrLm1* and *AvrLm2*. However, each of these avirulence genes is among the least frequently observed genes sampled in the population including *AvrLm4* and *AvrLm9.* In other published collections, the frequency of *AvrLm9* is low or non-existent (Zhang et al. 2016; Balesdent et al. 2005). This could be a reflection of the global distribution of the gene *AvrLm4-7,* which blocks the expression of *AvrLm9* (Ghanbarnia et al. 2018). The widespread detection of *AvrLm4-7* by PCR implies that most of the north Idaho collection (91%) will not be able to express a resistance response in the differential for the genes *AvrLm3* and *AvrLm9* (Ghanbarnia et al. 2018; Plissonneau et al. 2016). This was apparent during plant differential testing, as no isolates showed a response for *AvrLm3*, and only 3% showed a response for *AvrLm9*. Similarly, the gene *AvrLepR3* cannot be mapped in isolates containing *AvrLm1* when doing plant differentials as the gene confers avirulence for hosts containing *Rlm1* or *LepR3* (Larkan et al. 2013). The distribution of *Avr-1-2-4* is common among French and Canadian collections of *L. maculans* (Balesdent et al. 2006; Fernando et al. 2017).

Several cultivars/lines carry more than one *Rlm* gene, requiring multiple cultivars/lines to deduce the genes *AvrLm1* and *AvrLm2*. The genes *AvrLm5* and *AvrLm6* cannot be individually determined using plant differentials since the cultivar Cutlass contains both *Rlm5*

and *Rlm6* genes, making it critical to combine this information with PCR differentials that can distinguish *AvrLm5* and *AvrLm6* (Larkan et al. 2016).

Every *L. maculans* isolate tested positive for the genes *AvrLm7* and *AvrLm11*. The other commonly found avirulence genes (>90%) were *AvrLm5, AvrLm6 and AvrLepR1*. Due to their high frequency, the corresponding resistance genes in canola would be potential candidates for incorporation into PNW adapted varieties. With recent advancements in gene editing, isogenic lines containing resistance genes have been developed (Larkan et al. 2016). This is particularly useful for plant differentials, but could also be used to help with the introgression of novel resistance genes into existing adapted cultivars.

If the highest frequency genes in the population (>90%) were combined to predict a common representative race, it would result in *5-6-7-11-LepR1*. This race was the third most popular in the collection, with the others having the additions of *LepR2* and *LepR3*. Since *L. maculans* is found in many countries with widely varying agricultural practices, the genetic pool is tailored to each geographic region (West et al. 2001). North Idaho's most common race was *Avr-5-6-7-11-LepR1-LepR2*, occurring in 19% of all isolates sampled. In southern Manitoba, Canada, the most common race found was *Avr-2-4-5-6-7-11* (Fernando et al. 2017). In Alberta, on the western side of Canada, the most common race is *Avr-2-4-6-7* (Liban et al. 2016). The common races found in these two Canadian regions have adapted to include the genes *AvrLm2* and *AvrLm4* more frequently than isolates found in the north Idaho survey.

In 2006, large scale surveys of France showed the most popular individual genes as *AvrLm-1-4-5-6-7-8*. Isolates collected in the main oilseed producing regions did not contain *AvrLm1* due to a former resistance breakdown at the turn of the century (Balesdent et al.

2006). This occurred when producers overwhelmingly chose a canola variety that contained resistance gene *Rlm1,* accounting for 40% of oilseed production in 1998. This single variety reduced the frequency of the gene *AvrLm1* from 84% in 1997 to 13% in 2000 (Rouxel et al. 2003). *Leptosphaeria maculans* evolves quickly under selection pressure, which results in many combinations of genes (Sprague et al. 2006). Races are indicative of what genes are in the population but could easily vary between isolates. In the entire collection there were 38 distinct combinations of genes, but only six races contained more than five isolates. If single resistance genes were introgressed into existing cultivars, it could provide a useful tool in preventing the spread of black leg. If the group of genes *AvrLm5-6-7-11* was appropriately managed, it could encompass 79% of the collected isolates. Developing resistant cultivars would be a useful strategy only if the developed cultivars are part of a controlled regional rotation for north Idaho. If enacted without, it would most likely lead to producers choosing the highest yielding variety and increasing selection pressure on the existing population.

Although the use of resistant cultivars helps limit the damage caused by black leg, their effectiveness is limited because rapid breakdown of qualitative resistance. Between qualitative and quantitative resistance, host-pathogen interactions differ greatly. Qualitative refers to single gene-for-gene interactions that confer resistance as early as the seedling stage. The genes for qualitative resistance are usually dominant and can explain the majority of phenotypic variation for black leg resistance at the adult plant stage (Raman et al. 2013; Delourme et al. 2004). A major concern for using plants with qualitative resistance is the selective pressure they add against pathogen populations when resistant cultivars are not rotated. With reduced rotations and large populations of *L. maculans* interacting with a few

Rlm genes, resistance can breakdown in as little as three years with heavy selective pressure (Sprague et al. 2006; Brun et al. 2000).

There are currently no canola cultivars on the market with polygenic black leg resistance. This next advancement in plant breeding is crucial for providing durable resistance to black leg, but must be combined with other management strategies to limit this disease. Farming is difficult, and many of the choices producers are given can be assessed with a dollar value. Management choices like switching from conventional tillage to no-tillage operations can be costly and often do not improve yield for a number of years (Pittelkow et al. 2015). Convincing producers to change or extend crop rotations can be difficult if profit margins are on the line. Black leg control will not come from immediate financial decisions, but rather long-term management strategies.

Inland northwest crop rotations historically have been wheat, barley, and legume cropping systems that make use of intensive tillage practices. Over the last four decades, efforts have been made to shift antiquated production systems to conservation and no-till systems to reduce erosion (Kok et al. 2009). This shift is in part due to the extent of erosion on the Palouse, losing 40% of original topsoil in just the last century alone (Pimentel et al. 1995).

Tillage helps reduce overwintering inoculum because of faster residue degradation. However, having a strong rotation with a variety of crops also reduces disease pressure dramatically. When a Canadian study compared the incidence of black leg between no-till and conventional tillage, disease incidence was lower in a no-till system over the course of 5 years in a barley-canola rotation (Kutcher and Malhi 2010). Although conventional tillage still had a significant effect on reducing black leg incidence when a simple 2-year rotation (spring

canola, spring wheat) was compared to continuous cultivation, it became less effective with diverse rotations (Guo et al. 2005)..

Ideal rotations for canola would include a diverse system with crops such as barley, wheat, and legumes, avoiding consecutive plantings that might lead to a buildup in inoculum of *Brassica* pathogens (Johnston et al. 2005). Limited rotations that only utilize two crops will inevitably increase disease pressure, as residue left over from a previous crop could still be infectious. When grown in continuous monoculture or short rotations, canola residue does not have time to decompose before the next host crop. As a consequence, successive generations of *L. maculans* lead to increased pathogen population, and reduced the effectiveness of host resistance (Kutcher et al. 2013). The use of alternative crop rotations gives residue time to break down while limiting the population of host plants.

Management of this disease will fall on many shoulders, including researchers to survey for data, producers to diversify crop rotations, and companies to provide multiple lines of resistant cultivars. Modern farming practices including no-till, diverse crop rotation, and precision agriculture have already been proven to benefit producers in the long run. The way we treat these aspects of management likely plays an influential role in how each region experiences such an adaptive disease.

Because of its low disease severity on *Brassica* crops and weeds in the region, disease incidence alone will most likely not stir producers to enact progressive management strategies. Unless farmers are growing canola as a certified seed crop, black leg does not appear to be an immediate threat to *Brassica* crops in north Idaho. The inspection and treatment of canola seed before planting should continue as a mandatory preventative measure.

No scientific undertaking is without its hindsight. If the project was to be completed again, there are some suggestions that would have benefited this observational study. Sampling of black leg symptoms could have been reviewed earlier in the process so that the collection represented a larger geographical area. Most of the sampling took place along major roads and excluded fields that were inaccessible by roadway. By reviewing sample locations early on, more diverse sampling routes could be planned that better represent larger geographic regions within counties. In addition, asking growers about which cultivar they planted would have provided information about whether resistant varieties were grown or not. In this study, it was assumed that no producers were specifically growing canola cultivars that had black leg resistance.

When completing the differentials, genetic techniques were far more efficient, requiring less time and space to complete. Given the choice, I would have preferred to complete all the differentials by using PCR techniques, but not all avirulence genes in *L. maculans* had developed primers. The completion of plant differentials returned valuable information, but there is not currently a common global standard for cultivars or rating system. Given that the rating of phenotypic symptoms is subjective, a standard would help reduce variability in studies that complete plant differential tests.

This study helped establish a base line for observing the progression of *L. maculans* in north Idaho but could have benefited by understanding the disease's life cycle in our area. Both mating types were observed in north Idaho populations which confirms that the disease can reproduce sexually. What is largely unknown is at what time the sexual spores are released and consequently what time of year plants become infected. Spore traps are the next logical step in determining what time of year spore release occurs. Measuring the spore

release over the course of a few years could help predict at what time the most disease pressure occurs. This information could also be interpreted into a life cycle tailored to north Idaho, which would provide growers with specific information about how the disease proliferates in this area and at what time crops incur the most risk.

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Appendix A: Differential Results for North Idaho Collection

Results in this table represent the individual isolates analyzed from the 2016-2017 north Idaho survey. The county and town explain a general location of where the isolate was collected. Exact sampling locations can be found in Table 2.1. The first part of the isolate's name (A01) corresponds to the date and location the isolate was collected: A – 6/2/16, B – 6/16/16, C – 11/16/16, D – 5/12/17, E – 5/25/17, F – 6/5/17, G – 6/6/17. Samples with the designation PHL are from a collaborative collection maintained by Washington State University. The plant differential includes only the phenotypic results from the greenhouse screening, and the PCR differential only includes results from PCR analysis. The race is a combined result of both differentials, excluding unconfirmed results in parentheses such as (5-6). All avirulence genes discussed in the results belong to the designation *AvrLm* or *AvrLep.*

County	Town	Isolate	Plant Differential	PCR Differential	Race (Avr)
NPERCE	Lewiston	A01.1a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
NPERCE	Lapwai	A02.1a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
NPERCE	Lapwai	A02.4a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
NPERCE	Lapwai	A02.4b	(5-6)-7-LepR1	3-(4-7)-5-6-11	3-5-6-7-11-LepR1
LEWIS	Winchester	A04.1a	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6	3-5-6-7-LepR1-LepR2
LEWIS	Winchester	A04.1b	(5-6)-7-LepR1-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR3
LEWIS	Winchester	A04.2a	(5-6)-7-LepR1-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR3
LEWIS	Winchester	A04.2b	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2
LEWIS	Winchester	A04.3a	(5-6)-7-LepR1-LepR2	3-5-6-11	3-5-6-7-11-LepR1-LepR2
LEWIS	Winchester	A05.2a	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3
LEWIS	Winchester	A05.2b	1-(5-6)-7-LepR1-LepR2-(LepR3)	3-(4-7)-5-6-11	1-3-5-6-7-11-LepR1-LepR2

LEWIS	Winchester	A05.3a	(5-6)-7-LepR1	3-(4-7)-5-6-11	3-5-6-7-11-LepR1
IDAHO	Ferdinand	A07.1a	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2
IDAHO	Ferdinand	A07.1b	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Ferdinand	A07.2a	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Ferdinand	A07.2b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Fenn	A08.1a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Fenn	A08.1b	2-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	2-3-5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	A09.1a	(5-6)-7-LepR1-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR3
IDAHO	Denver	A09.1b	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Denver	A09.2a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	A09.2b	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	A09.2c	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	A09.3a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Denver	A09.3b	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	A09.4a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	A09.4b	4-(5-6)-7-LepR1	(4-7)-5-6-11	4-5-6-7-11-LepR1
IDAHO	Denver	A09.5a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	A09.5b	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Denver	A09.6a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	A09.6b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	A10.1a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	A10.2a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Denver	A10.2b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	A10.3a	(5-6)-7-LepR1-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR3
IDAHO	Denver	A10.3b	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Grangeville	A11.1a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Grangeville	A11.1b	2-(5-6)-7-LepR1-LepR3	(4-7)-5-6-11	2-5-6-7-11-LepR1-LepR3

IDAHO	Grangeville	A11.2a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Grangeville	A11.2b	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Grangeville	A11.3a	(5-6)-7-LepR1-LepR2	5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Nezperce	A12.1a	(5-6)-7-9-LepR1-LepR2	(4-7)-5-6-11	5-6-7-9-11-LepR1-LepR2
IDAHO	Nezperce	A12.1b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Nezperce	A12.2a	(5-6)-7-LepR1-LepR2	(4-7)-11	7-11-LepR1-LepR2
IDAHO	Nezperce	A12.2b	2-(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	2-5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Nezperce	A12.3a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Nezperce	A12.3b	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Nezperce	A12.4a	(5-6)-7-LepR1-LepR2	(4-7)-5-11	5-7-11-LepR1-LepR2
IDAHO	Nezperce	A12.4b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Nezperce	A12b.1a	*L. biglobosa*	5-6	5-6
IDAHO	Nezperce	A12b.1b	*L. biglobosa*	5-6	5-6
NPERCE	Leland	B02.1a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
NPERCE	Leland	B02.1b	(5-6)-7-LepR1-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR3
NPERCE	Leland	B02.2a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
NPERCE	Leland	B02.2b	4-(5-6)-7-LepR1-LepR2	(4-7)-5-11	4-5-7-11-LepR1-LepR2
NPERCE	Leland	B02.2c	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
NPERCE	Leland	B02.3a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-11	5-7-11-LepR1-LepR2-LepR3
NPERCE	Cavendish	B03.2a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
NPERCE	Cavendish	B03.3b	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
LEWIS	Craigmont	C03.1a	(5-6)-7-LepR1-LepR2	(4-7)-5-11	5-7-11-LepR1-LepR2
LEWIS	Craigmont	C03.1b	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2
LEWIS	Craigmont	C03.1d	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
LEWIS	Craigmont	C03.1e	(5-6)-7-LepR1-LepR2	(4-7)-5-11	5-7-11-LepR1-LepR2
LEWIS	Craigmont	C03.1f	(5-6)-7-LepR1-LepR2	5-11	5-7-11-LepR1-LepR2

LEWIS	Craigmont	C03.1g	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2
LEWIS	Craigmont	C03.1h	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
LEWIS	Craigmont	C03.1i	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Fenn	C04.1a	*L. biglobosa*	no amplification	
IDAHO	Fenn	C04.3a	*L. biglobosa*	(4-7)	(4-7)
IDAHO	Fenn	C04.4b	*L. biglobosa*	no amplification	
IDAHO	Fenn	C04.5b	*L. biglobosa*	no amplification	
IDAHO	Fenn	C04.6b	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
LATAH	Moscow	D01.1a	*L. biglobosa*	no amplification	
LATAH	Moscow	D01.1b	*L. biglobosa*	no amplification	
LATAH	Moscow	D01.3a	*L. biglobosa*	no amplification	
LATAH	Moscow	D02.1b	*L. biglobosa*	no amplification	
LATAH	Moscow	D02.2a	1-(5-6)-7-LepR1-LepR2-(LepR3)	1-(4-7)-6	1-6-7-LepR1-LepR2
IDAHO	Fenn	D03.1a	4-(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2
IDAHO	Fenn	D03.2a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Fenn	D03.2b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Fenn	D03.3a	4-(5-6)-7-9-LepR1-LepR2-LepR3	5-6-11.	4-5-6-7-9-11-LepR1-LepR2-LepR3
IDAHO	Fenn	D03.3b	1-(2-3)-4-(5-6)-7-9-(LepR3)	5-6-11.	1-4-5-6-7-9-11
IDAHO	Fenn	D03.4a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Grangeville	D04.1a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Grangeville	D04.1b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5	5-7-LepR1-LepR2-LepR3
IDAHO	Grangeville	D04.2a	1-(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	1-5-6-7-11-LepR1-LepR2
IDAHO	Grangeville	D04.2b	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Grangeville	D04.3b	(5-6)-7-LepR1-LepR2-LepR3	5	5-7-LepR1-LepR2-LepR3
IDAHO	Grangeville	D04.4a	(5-6)-7-LepR1	3-(4-7)-5-6-11	3-5-6-7-11-LepR1

IDAHO	Grangeville	D04.4b	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Grangeville	D05.1a	(5-6)-7-9-LepR1-LepR2	(4-7)-5-6-11	5-6-7-9-11-LepR1-LepR2
IDAHO	Grangeville	D05.2a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	D06.1a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Denver	D06.1b	4-(5-6)-7-LepR1-LepR2-(LepR3)	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2
IDAHO	Denver	D06.2a	2-(5-6)-7-LepR1-LepR2	(4-7)-5-6	2-5-6-7-LepR1-LepR2
IDAHO	Denver	D06.2b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	D07.1a	(5-6)-7-LepR1	(4-7)-5-11	5-7-11-LepR1
IDAHO	Denver	D07.1b	(5-6)-7	(4-7)-5-6-11	5-6-7-11
IDAHO	Denver	D07.2a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Denver	D07.2b	(5-6)-7-LepR1-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR3
IDAHO	Denver	D07.3a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	D07.3b	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	D08.1a	(5-6)-7-LepR1-LepR2	(4-7)-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	D08.1b	(5-6)-7-LepR1	(4-7)-5-11	5-7-11-LepR1
IDAHO	Denver	D08.1c	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Denver	D08.1d	4-(5-6)-7	(4-7)-6-11	4-6-7-11
IDAHO	Denver	D08.2a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
IDAHO	Denver	D08.2b	7-LepR2	(4-7)-5-6-11	5-6-7-11-LepR2
IDAHO	Denver	D08.2c	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	D08.3a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
IDAHO	Denver	D08.3b	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
LEWIS	Craigmont	D10.1a	(5-6)-7-LepR1-LepR2	(4-7)-6-11	6-7-11-LepR1-LepR2
LEWIS	Craigmont	D10.2a	(5-6)-7	(4-7)-6-11	6-7-11.
LEWIS	Craigmont	D12.2b	(5-6)-7-LepR2	(4-7)-6-11	6-7-11-LepR2
SPOKN	Fairfield	E02.1a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-6-11	6-7-11-LepR1-LepR2-LepR3
SPOKN	Fairfield	E02.1b	(5-6)-7-LepR1-LepR2	(4-7)-6-11	6-7-11-LepR1-LepR2
LATAH	Genesee	F01.1a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
LATAH	Genesee	F01.2a	(5-6)-7	(4-7)-5-6-11	5-6-7-11
LATAH	Genesee	F01.3a	(5-6)-7-LepR2	(4-7)-5-6-11	5-6-7-11-LepR2

LATAH	Genesee	F01.4a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
LATAH	Genesee	F02.1a	(5-6)-7	(4-7)-5-6-11	5-6-7-11
NPERCE	Lapwai	G01.1a	(5-6)-7-LepR1	11	7-11-LepR1
NPERCE	Lapwai	G02.1a	(5-6)-7	(4-7)-6-11	6-7-11
NPERCE	Lapwai	G02.1b	(5-6)-7-LepR1	5-6-11	5-6-7-11-LepR1
NPERCE	Culdesac	G03.1a	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
NPERCE	Culdesac	G03.2a	(5-6)-7-LepR1	5-6-11	5-6-7-11-LepR1
NPERCE	Culdesac	G03.2b	(5-6)-7	(4-7)-5-6-11	5-6-7-11
NPERCE	Culdesac	G03.3a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
NPERCE	Culdesac	G04.2a	1-4-(5-6)-7-LepR1-LepR2	3-6-11	1-3-4-6-7-11-LepR1-LepR2
NPERCE	Culdesac	G04.3a	(5-6)-7-9-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-9-11-LepR1-LepR2-LepR3
NPERCE	Culdesac	G04.3b	(5-6)-7-LepR1-LepR2-LepR3	5-11	5-7-11-LepR1-LepR2-LepR3
LATAH	Kendrick	G05.1a	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2
LATAH	Kendrick	G05.2a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
LATAH	Kendrick	G05.2b	(5-6)-7	(4-7)-5-6-11	5-6-7-11
LATAH	Kendrick	G05.3a	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3
LATAH	Kendrick	G05.3b	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3
LATAH	Troy	G06.1a	(5-6)-7-LepR1-LepR2	3-5-6-11	3-5-6-7-11-LepR1-LepR2
NPERCE	Lewiston	PHL010	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Camas Prairie	PHL026	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Camas Prairie	PHL029	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1
IDAHO	Camas Prairie	PHL039	*L. biglobosa*	no amplification	