Oomycete Potato Pathogens: Characterization, Diagnostics and Disease Management

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Abstract

Oomycete pathogens such as *Phytophthora infestans, P. erythroseptica,* and *Pythium ultimum* infect potato in the field and storage, causing significant yield and storage losses. These oomycete pathogens are major constraints to profitable potato production. Late blight caused by *Phytophthora infestans* is a severe problem in most major potato production countries. Breeding for late blight resistance would be the best way to control late blight in developing countries due to the reduced exposure to fungicides, less residues in food, land, and water, and reduced cost of fungicides. The Feed the Future Biotechnology Potato Partnership (FtFBPP) was formed in 2015 as a 5-year, multi-institutional cooperative agreement with USAID to introduce bioengineered 3 R-gene potato cultivars into Bangladesh and Indonesia. Before releasing the bio-engineered potato cultivars, we need to know the *P. infestans* populations in Bangladesh and Indonesia. So, to determine the genotypes present in these countries, 160 samples from Bangladesh and 146 samples from Indonesia were collected and characterized based on one-step multiplex microsatellite markers, mating type, mitochondrial haplotype, and metalaxyl-M sensitivity test. Microsatellite analysis revealed that EU_13_A2 (metalaxyl-M resistant genotype) caused late blight outbreaks in Bangladesh and EU_2_A1 (metalaxyl-M sensitive genotype) in Indonesia. We also found a large sub-clonal variation of EU_13_A2 in Bangladesh and other unique genotypes in Indonesia. These findings will serve as a baseline to inform the development of integrated strategies to extend the efficacy and durability of the USAID FtFBPP 3 R-gene potato cultivars developed for release in Bangladesh and Indonesia.

Late blight can destroy potato fields in a few days under conducive environmental conditions if proper control measures are not applied. Early detection of *P. infestans* and the identification of genotypes can ensure the timely implementation of an optimum disease management strategy. Recently, loop-mediated isothermal amplification (LAMP) assays have become more widely used for the rapid on-site detection of *P. infestans*. We developed a new LAMP assay using the *ypt1* gene. Our LAMP assay was tested for sensitivity, specificity, and detection limit and compared with other previously developed assays. Our LAMP assay was superior in sensitivity and specificity over other LAMP assays, since it did not cross-react with species closely related to *P. infestans,* except for *P. andina* and *P. ipomoeae*. *Phytophthora ipomoeae* was easy to distinguish because it amplified very late in the reaction.

The lower limit of detection (LOD) of our LAMP assay was determined to be 1 pg/µL (LAMP run for 25 min) for pure culture. Our LAMP assay can be used in the field to detect *P. infestans* so that growers can use proper control measures to reduce the loss caused by this pathogen.

Potatoes are kept in storage for a few weeks to several months after harvest based on the intention of use or market availability. Potato tubers contaminated or infected in the field during the growing season or at harvest are vulnerable to many storage rot diseases such as Pythium leak and pink rot. Due to limited chemical control measures, the quality and health of tuber are compromised in storage, causing significant economic loss. Much research has been focused on different cultural and epidemiological factors of the storage diseases of potatoes. The disease severity in tubers infected with *Pythium ultimum* was reported to be higher at high storage temperatures. A systematic study was conducted to determine the level of disease severity at four pre-storage temperatures (15°C, 20°C, 25°C, and 30°C), and two storage temperatures, 8.8°C, and 12.8°C, to emulate the conditions in commercial storage and temperatures at harvest and prior to storage. In our findings, the tubers incubated at 15°C and stored at 8.8°C had the lowest disease severity and incidence followed by those incubated at 20°C prior to storage. Similarly, disease severity and incidence were lower in tubers incubated at 15°C and stored at 12.8°C. In artificial inoculation experiments, tubers showed symptoms much earlier than the naturally infected tubers. However, the trend of disease development over time was similar. Another study found that *Pythium* inoculum as low as one spore was enough to initiate disease. In summary, a temperature higher than 20°C during harvest and storage temperatures of 12.8°C, high inoculum density in the field, wounding, and bruising lead to high disease severity in the storage.

Seven commercially available in-season foliar fungicides were evaluated for control of Pythium leak and pink rot in storage. Potato tubers harvested and stored at 12.8°C for two to three months were challenge-inoculated and stored at 18°C for 30 days. The volume covered by the Pythium leak or pink rot symptoms was recorded for disease severity in percentage. None of the fungicides tested significantly reduced the Pythium leak in storage in all three years except in 2018 (field 113). Both rates of phosphorous acid significantly reduced the Pythium leak in storage. In pink rot trials, both rates of phosphorous acid, cyazofamid, fluazinam, oxathiapiprolin, and mefenoxam significantly reduced the pink rot in storage.

Therefore, the findings of this study can be added to an integrated disease management strategy to reduce the loss in storage.

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1 LITERATURE REVIEW

1.1 Introduction

1.1.1 The potato crop

The potato (*Solanum tuberosum* L.) is thought to have originated in the Andes Mountains range in southern Peru and northwestern Bolivia between 8000 and 5000 BC (Bradshaw & Ramsay, 2009; Hawkes & Francisco-Ortega, 1993). Historically, the inhabitants of the Andes in South America were the first people to domesticate the potato (Brown & Henfling, 2014; International Potato Center, 2008). These inhabitants were interested in domesticating the wild plants, which had energy-rich starchy underground tubers (Brown & Henfling, 2014; CIP, 2008). Since the energy-rich potato was easily storable and transportable, the Andes inhabitants started collecting thousands of types of potatoes from the wild and domesticated them (Brown & Henfling, 2014; International Potato Center, 2008). Later, when the Spanish conquistadores arrived in the Andean society, it is believed that the potato was introduced to the Canary Islands of Spain by the Spanish around 1567 (Hawkes & Francisco-Ortega, 1993). The potato became popular in Spain as a health-restoring food as it was a lifesaver for sick people (Salaman & Burton, 1985). The Protestant Waldensians became potato farmers and spread the potato in northern Europe (Brown & Henfling, 2014). When fleeing from Roman Catholics, Waldensians brought the potatoes to the northern European countries such as France, Germany, and The Netherlands (Reader, 2009). During the political and religious wars in Europe, the potato was spread to European gardens and fields because potatoes could remain in the soil and were not destroyed like cereal crops (De Jong, 2016; Salaman & Burton, 1985).

The potato became popular in Europe mainly because it was more productive than other crops in a conducive climate, easy to grow, and potatoes were not taxed at the time (Brown & Henfling, 2014; Reader, 2009). The population of Europe had increased from 140 million to 266 million within 100 years due to the increase in the food supply, mainly potatoes and maize (Langer, 1975; De Jong, 2016). By the 1750-the 1850s, the potato was well established in northern European countries such as France, Belgium, the Netherlands, Germany, Denmark, Poland, and Russia (De Jong, 2016). The potato had also reached the

fields of Irish farmers by that time, and by the 1790s, the potato had become the staple food of the Irish (Salaman & Burton, 1985).

From 1750 to the 1850s, most of the land in Ireland was owned by English landlords. Irish farmers had to produce enough food from their limited lands. The potato became the miracle crop for the Irish because they could make enough food to support their families from their small plots, and the potato was tax-free from the British landlords (Brown & Henfling, 2014). Due to the temperate climate, potato production flourished in Ireland, and by the end of the 17th century, the potato became the staple food for the Irish people (Burton, 1948). The Irish had a good food supply, and the population of Ireland significantly increased compared to the other European countries from 1750 to 1841 (De Jong, 2016; Langer, 1975; Salaman & Burton, 1985). However, Irish people were overly dependent upon the potato. In the 1840s, the potato fields of Irish farmers were hit hard by an unknown disease that destroyed most of their potato crops and the stored potatoes (Woodham-Smith, 1962). Potato production was severely impacted by the unknown disease, which led to a famine known as the "Great Hunger" (Woodham-Smith, 1962). One million people starved to death in the famine, and about the same number of people emigrated to North America (Woodham-Smith, 1962). Today, the famine is well known as the "Irish Famine." After much research, the disease was found to be caused by a pathogen which was initially named as *Botrytis infestans* and later *Phytophthora infestans* (Ribeiro, 2013). The study of late blight and the discovery of *P. infestans* formally started the discipline of plant pathology.

There are no definitive records on how the potato was introduced in North America. It is believed the potato was introduced in New Hampshire by a group of Irish immigrants from Northern Ireland in 1719. However, there is other evidence that the potato was mentioned before the Irish immigrants (De Jong, 2016). The potato was the staple diet for the gold miners in California in the 1800s (Burke, 2007). The potato was first grown in Idaho in 1837 by Henry Spalding and succeeded mainly due to the immigration of Mormons to Idaho, gold miners, and the suitability of the Burbank cultivar in Idaho growing environment (Davis, 1992). In the United States, *P. infestans* was first discovered in New York and Philadelphia in 1843 (Stevens, 1933).

By the late 17th century, potatoes were distributed to Asian countries by European colonists (Spanish, Dutch and British; Bradshaw & Ramsay, 2009). The potato was distributed from Europe to tropical and subtropical countries in the 19th century (Beukema & van der Zaag, 1990). The modern cultivated potato is thought to have been selected from the *Solanum brevicaule* complex (Spooner et al, 2005). Currently, potatoes are produced in 149 countries worldwide in many different environments from sea level to 4000 m (International Potato Center, 2010; Hijmans, 2001).

1.2 Potato production

Potatoes can be grown anywhere from 65°N to 47°S and from sea level to as high as 4000 m (Hijmans, 2001). However, a temperate climate is most suitable for potato production (Hijmans, 2001). The total potato production in southern hemisphere is only 6.9% and there are only four countries in the southern hemisphere out of 26 that produce potatoes in more than 100,000 ha worldwide (Hijmans, 2001). Potatoes are grown as a summer crop (latitude between 44°N and 58°N) or winter crop (23°N and 34°N) depending upon the latitude of the northern hemisphere (Hijmans, 2001). The extreme temperature during potato production is not considered good. Potato production regions should be frost-free for at least 100 consecutive days with broad day/night temperature differentials and an average annual rainfall of 50 to 60 cm (Pavek, 2014). Potatoes grow best in medium to coarse-textured soil such as silt loams, loams, sandy loams, and sand with pH between 6.0 and 7.5 (Magdoff & van Es, 2000; Pavek, 2014). Potatoes are vegetatively propagated, and whole tubers (single drop) or cut seed pieces are planted to start the crop (Pavek, 2014).

1.2.1 Global potato production

The potato is a solanaceous crop and is consumed as a staple food around the globe as it provides a good source of carbohydrates, proteins, vitamins, and minerals (Bradshaw & Ramsay, 2009; Niederhauser, 1993). The potato is the fourth most important crop in the world after wheat, maize, and rice (FAO, 2021). The potato is popular because of its adaptability to different environments, ease of vegetative propagation, production, and richness in nutritional contents (Fiers et al, 2012; Hijmans, 2001, Secor & Rivera-Varas,

2004). Potato production has increased mainly due to the expansion of the area in Asian countries (CIP, 2010; Guenthner, 2010).

The potato is grown worldwide in many climate and soil conditions (FAO, 2021). Approximately 93% of potatoes are grown in countries in the northern hemisphere (Hijmans, 2001). In 2019, a total of 370 million metric tonnes of potatoes were harvested from 17 million hectares (ha) of land worldwide (FAO, 2021). China is among the top potato producers with 92 million metric tonnes, followed by India with 50.2 million metric tonnes, Russia with 22.1 million metric tonnes, Ukraine with 20.3 million metric tonnes, and the USA with 19.2 million metric tonnes (FAO, 2021). Global potato production has undergone a considerable shift from Europe to Asian countries. In the early 1990s, countries in Europe, North America, and the Soviet Union grew and consumed most potatoes. However, in the recent years, most potatoes have been produced in China, India, and other Asian countries. Potato production in Asia, Africa, and Latin America has increased from less than 30 million metric tons in the 1960s to 165 million metric tons in 2007 (www.potatopro.com).

In Asian countries, Bangladesh is the third-largest country in potato production after China and India, producing 9.7 million metric tons (FAO, 2021). In Bangladesh, the potato is the third most important crop after rice and wheat (Siddique et al, 2015). Bangladesh harvested 9.7 million tonnes of potatoes from 468,000 ha in 2019 (FAO, 2019). The average yield of potatoes in Bangladesh is 19.65 tonnes/ha. The yield is significantly lower than the average yield in developed countries and is mainly due to low-yielding cultivars, diseases, and pests (BBS, 2018; Hossain et al, 2008). Indonesia is one of the largest potato producers in southeast Asia (FAO, 2008). Potatoes are grown in Indonesia by small-scale farmers throughout the archipelago, mainly in the highlands (800 – 1800 m; FAO, 2008). Potato production in Indonesia has increased significantly since 1969, mainly due to an increase in the total production area and increased productivity to a lesser extent (Adiyoga et al, 1999). Indonesia harvested 1.3 million metric tons of potatoes from 68 thousand hectares in 2019 (FAO, 2019). The average yield of potatoes was 15.4 tonnes/ha, which is significantly lower compared to developed countries (Indonesia Potato Pro, 2017). In Indonesia, more than 50 percent of potatoes are grown on the island of Java, with 37 percent of those produced in West Java (Adiyoga et al, 1999).

1.2.2 Potato production in the US

The United States is the fifth major potato-producing country globally, producing 21.5 million metric tonnes harvested from 379 thousand hectares (ha; USDA, NASS 2020). In 2019, the gross potato production value was $4.2 billion (FAO, 2020). In 2019, the top ten potato-producing states in the United States were Idaho, Washington, Wisconsin, Oregon, Michigan, Colorado, North Dakota, Minnesota, California, and Maine. Among these states, Idaho is the leading state in potato production in the United States. The southeastern part of Idaho is the major potato production area (Madsen & Otter, 2011). In 2019, 124,643 ha of potatoes were planted in Idaho, with a total production of 6.7 million metric tonnes and a production value of $975 million. This amounts to almost one-third (~ 31.2%) of US total potato production (USDA, NASS 2020). In 2016, the main cultivars were grown in Idaho (harvest by acreage) were Russet Burbank (51.3%), Russet Norkotah (16.7 %), and Ranger Russet (13.1%, Idaho Potato Pro, 2019).

During summer, potatoes are grown in Idaho, Washington, Oregon, Wisconsin, North Dakota, Minnesota, Michigan, and Maine, as these states are in the temperate climate zone (latitude between 45°N and 57°N; Hijmans, 2001; Pavek, 2014). Potatoes are grown in winter in Texas, Arizona, and Florida as these states lie in subtropic climate zone (latitude between 23°N and 34°N; Hijmans, 2001; Pavek, 2014). Potatoes in the USA are commercially produced from disease-free certified seed using a high level of mechanization from planting to harvesting (Davidson & Xie, 2014). Winter potato crops are grown from November to February, while summer potato crops in temperate regions are grown from April to early June (Pavek, 2014). The soil is tested for nutrients, and pre-plant fertilizers are added before planting (Pavek, 2014). Seed potatoes are planted either as a single drop or cut seed pieces when the soil temperature exceeds 7°C (Davidson & Xie, 2014; Isleib & Thompson, 1959). Since cutting and handling operations can expose seed pieces to pathogens, they are treated with fungicides before or at planting to protect them from seed and soil-borne pathogens (Lambert et al, 1998). Potato cultivars are chosen based on soil type and market demand (Pavek, 2014). For example, Idaho has sandy loam soil and is ideally suited for Russet-type potato cultivars. Potatoes are usually planted in rows which makes cultivation practices and harvesting easier. Potato size is mainly controlled by adopting close plant spacing. The optimum amount of water for potato production is maintained at 60 to 85% of

available soil water at a depth of 0 to 45 cm (King, Stark & Neibling, 2020). Potatoes need maximum available soil water (65-85%) during tuberization and tuber bulking stage and less towards senescence (60%; King, Stark & Neibling, 2020). Macronutrients such as nitrogen, phosphorous, potassium, magnesium, and sulfur, and micronutrients such as boron, copper, iron, chloride, manganese, and zinc are required for potato production and applied based on the soil type and recommended dose. Any fungicide sprays are planned based on the calendar schedule in the USA. The vines of potatoes are killed 14 to 21 days before harvest to minimize the skin injury of potatoes during harvest. Potatoes are harvested, and tubers are stored in storage facilities maintained with appropriate temperature and humidity until use.

1.2.3 Major constraints of profitable potato production

Several biotic, abiotic, and technical factors limit profitable potato production. Technical factors such as lack of certified seeds, unsuitable varieties, no fertilizers and pesticides, irrigation facilities, and mechanization in the potato production system play a significant role in less potato production in developing countries. Other environmental problems are drought, over-humid soil, and poor soil structures. The biotic factors such as weeds, disease, insects, and pests are other significant constraints in potato production. Besides biotic and non-biotic problems, lack of suitable storage, price instability, and lack of market impedes profitable potato production (Manrique, 1993; Maldonado et al, 1998; Taylor & Dawson, 2021).

After potatoes are planted in the field, they face numerous diseases at every growth stage, from emergence to harvest and even in storage (Alyokhin, Vincent & Giordanengo, 2012; Kirk & Wharton, 2014; Stevenson, Loria, & Franc, 2001). Potatoes are stored for an extended period for continuous supply to consumers, processing industries, or for seed purposes (Paul, Ezekiel & Pandey, 2016). The lengthy storage period increases the risk of tuber rot due to the diseases that affect tubers in storage. Diseases caused by fungal, bacterial, viral, and oomycete pathogens have a negative impact on sustainable and profitable potato production (Kirk & Wharton, 2014; Powelson & Rowe, 2008; Secor & Gudmestad, 1999). Several potato diseases limit profitable potato production, including fungal diseases such as early blight, white mold, gray mold, rhizoctonia stem canker, silver scurf, powdery scab, black dot, powdery mildew, verticillium wilt, and dry rot. Bacterial diseases include blackleg,

bacterial soft rot, brown rot, ring rot, common scab, and zebra chip. Viral diseases caused by PVY, potato leafroll virus, tobacco rattle virus, potato mop-top virus is also a problem for seed potato growers (Charkowski et al, 2020; Kirk & Wharton, 2014; Kreuze et al, 2020; Taylor & Dawson, 2021). All these diseases decrease the yield, rot the tubers in storage or the field and lower the quality of potatoes for seed and consumption. These diseases increase the cost of potato production. Because to control these diseases, growers need costly fungicide sprays, certified seeds, disease forecasting and monitoring programs, resistant cultivars to prevent these diseases.

Amongst all the potato diseases and pathogens, the oomycete pathogens, *Phytophthora infestans* (Mont. de Bary), *Phytophthora erythroseptica* (Pethybr.), and *Pythium ultimum* (Trow) are amongst the most destructive (Secor & Gudmestad, 1999). The estimated loss from these pathogens is in the billions of dollars due to poor management of disease, the emergence of fungicide insensitive isolates/genotypes/biotypes, lack of resistant cultivars, lack of proper disease forecasting systems, lack of knowledge about the epidemiology of the pathogens and lack of timely and precise diagnostics tools. The estimated loss (yield loss and fungicide cost) due to late blight alone exceeds more than $6.7 billion annually (Haverkort et al, 2008).

The integrated management of the major potato diseases includes the availability of certified seeds, fertilizers, and pesticides on time, proper selection of well-drained sites for potato production, and availability of irrigation. Scouting and monitoring of disease, insects, and pests should be done routinely, and control should be done on time. Harvesting should be done after the skin set. Proper storage temperature and humidity should be maintained in the storage facilities.

1.3 Oomycete pathogens

Oomycetes are eukaryotic plant pathogens classified in the kingdom Chromista (Stamenopila) based on the evolutionary phylogeny (Cavalier-Smith, 1986; Hawksworth et al, 1995; Rossman, 2006). The closest relatives of oomycetes are heterokont, biflagellate, brown algae (Cavalier-Smith, 1986; Fry & Grünwald, 2010; Hawksworth et al, 1995; Rossman & Palm, 2006). The oomycetes were confused with the true fungi because of their filamentous

growth. Therefore, oomycetes were previously included in the kingdom of Mycetae (Erwin & Ribeiro, 1996). Oomycetes were previously included in Phycomycetes and Zygomycetes because they had one common feature: the lack of septa in their mycelia (Erwin & Ribeiro, 1996). However, oomycetes have many distinct characteristic morphological features different from true fungi.

The phylum oomycota is characterized by spherical oospores due to sexual reproduction (Trigiano, Ament & Lamour, 2007). Besides oospores, oomycetes produce asexual propagules, namely sporangia and zoospores. The diploid oospores are produced as zygotes after the fertilization of haploid oospheres (Rossman & Palm, 2006). The oospore has a double cell wall that helps resist freezing and other detrimental conditions in the soil or plant debris (Drenth, Janssen & Govers, 1995; Hegnauer & Hohl, 1978). Oospores can survive for several years in plant debris or free in soils and become a primary source of inoculum (Lehtinen & Hannukkala, 2004). Other characteristics of oomycetes are hyphae being coenocytic, sexual reproduction through gametangial contact, and most of the life cycle in the form of diploid (Trigiano, Ament & Lamour, 2007).

1.3.1 Taxonomy of the genus *Phytophthora*

Phytophthora is derived from a Greek word that means “Phyto” plant and “Phthora” destroyer (Erwin & Ribeiro, 1996). German mycologist Anton de Bary first described the name Phytophthora in 1876. Anton de Bary first described white sporulation on infected potato leaves as the causal organism of late blight and *Phytophthora infestans* as a causal organism of late blight that led to the Irish famine in 1845 (Schumann & D’Arcy, 2000; Zentmyer, 1983). He is considered the father of the plant pathology discipline (Hansen, Reeser & Sutton, 2012). *Phytophthora infestans* were previously identified as *Botrytis infestans* or *Peronospora infestans* (Ho, 2018).

The genus *Phytophthora* is a member of the order Peronosporales of the class Oomycetes (Beakes & Sekimoto, 2009). The Peronosporales order is sub-divided into Pythiaceae, Peronosporaceae, and Albuginaceae families based on the type of sporangia and sporangiophores of the oomycetes (Trigiano, Ament & Lamour, 2007). The genus *Phytophthora* is placed in the Peronosporaceae family due to its differentiated sporangiophores, distinct (conspicuous) periplasm, and obligate parasites nature (Ko et al, 2010). The morphological characteristics of the genus *Phytophthora* include motile

biflagellate (one tinsel and another whiplash flagellum) zoospores, the cell wall composed of cellulose, and Beta-1-6 and 1-3-glucan, non-septate hyphae, and diploid lifestyle (Fry, & Grünwald, 2010; Randall et al, 2005; Rossman & Palm, 2006). One of the distinct morphological features of *Phytophthora* is that they produce antheridia and oogonia as gametangia, in which meiosis occurs before fertilization (Fry, 2008; Sansome, 1965). Another unique feature of the genus *Phytophthora* is they do not produce sterols. However, they need to obtain beta-hydroxy sterols for sporulation from an exogenous source (Elliot, 1983).

The Phytophthora species are either biotrophic or hemibiotrophic but may also survive as saprophytes (Lamour & Kamoun, 2009). The hemibiotrophic species of Phytophthora initially behave as biotrophic pathogens in the infected host but act as a necrotrophic pathogen in the later tissue colonization stage (Shibata, Kawakita, & Takemoto, 2010). *Phytophthora infestans* are considered obligate hemibiotrophic pathogens under natural and agricultural conditions (Fry, 2008).

Around 59 *Phytophthora* species have been compiled by Erwin & Ribeiro (1996) in their *Phytophthora* diseases worldwide book, and 46 species by Waterhouse, Newhook & Stamps (1983). There are many taxonomic keys to categorize species of *Phytophthora*. However, the taxonomic key produced by Waterhouse (1963) is widely accepted. The taxonomic key includes a series of morphological characteristics by which the *Phytophthora* species are categorized into six groups (I-VI; Erwin & Ribeiro, 1996). The most common morphological characteristics taken into consideration while classifying the *Phytophthora* species are antheridium amphigynous or paragynous (attached to the side of oogonium), sporangium morphology (papillate or not or semi), presence or absence of caducity (shedding of sporangium at maturity), the proliferation of sporangia, branching of sporangiophores, presence or absence of chlamydospores, presence or absence of hyphal swelling, and maximum temperature for growth (Waterhouse, 1963; Stamps et al, 1990; Erwin & Ribeiro, 1996).

Recently, molecular approaches have added the potential to study the species in-depth, resulting in the discovery of more species (Hansen, Reeser & Sutton, 2012). There were approximately 114 *Phytophthora* species formally described by the end of 2011 (Hansen, Reeser & Sutton, 2012). The new DNA sequencing, the taxonomic concept for the genus, and analytical tools have provided the advantages of classifying the species of *Phytophthora* in

different clades based on molecular phylogeny (Blair et al, 2008; Cooke et al, 2000; Martin, Blair & Coffey, 2014; Yang, Tyler & Hong, 2017). The dichotomous key has recently been used with PCR-based DNA fingerprinting techniques (Gallegly & Hong, 2008). A comprehensive molecular phylogeny of *Phytophthora* has classified *Phytophthora* species into ten different clades (Yang, Tyler & Hong, 2017). For example, *P. infestans*, *P. andina*, *P. ipomoeae, P. mirabilis*, and *P. phaseoli* are grouped in clade one and subclade 1c based on molecular phylogeny. These species in subclade 1C produce semi papillate (small outgrowth tip) sporangia (Yang, Tyler & Hong, 2017). The traditional morphological taxonomy categorizes *P. infestans* in group IV (Waterhouse, 1963). There are many genotypes or clonal lineages and their variants within the species. For example, *P. infestans* has many genotypes such as US-1, US-8, US-22, US-23, US-24 in the United States and EU_1, EU_6, EU-13, EU_33 in the European countries.

The most notorious *Phytophthora* species recorded is *P. infestans* because, in the mid-19th century, the pathogen wiped out the potato crops in Ireland, causing a devastating potato famine (Andrivon, 1995). In the Irish famine, one million people were starved to death, and the same number of people were forced to leave Ireland and immigrate to North America (Abad & Abad, 1997). *Phytophthora infestans* is thought to have originated in the Toluca Valley of Mexico (Goodwin, Drenth & Fry, 1992; Goss et al. 2014; Grünwald & Flier, 2005) or South America (Abad & Abad 1997; Adler et al, 2004; Ristaino, 2002; Saville, Martin & Ristaino, 2016; Yoshida et al, 2013) and later spread around the globe.

Phytophthora cinnamomi is another destructive *Phytophthora* species that cause disease in more than 1,000 species of plants impacting forest ecosystems and crops. Chestnut is susceptible to *P. cinnamomi,* and it has wiped out the chestnut on conducive environments (Hansen, Reeser & Sutton, 2012). Other notable *Phytophthora* invasive species are *P. ramorum*, *P. kernoviae*, *P. alni* which have a substantial ecological and economic impact. In a study, Scott et al (2019) had included 12,500 disease reports caused by the genus *Phytophthora* worldwide.

1.3.2 Taxonomy of the genus *Pythium*

The genus *Pythium* was first created by Pringsheim in 1858. *Pythium* produces spherical and filamentous sporangia; hence it was proposed to differentiate species into two genera (Ho, 2018). Many debates created different infrageneric taxa based on morphological structures (Ho, 2018). However, the genus *Pythium* was adopted and conserved as Waterhouse (1968) described. The family Pythiaceae was created by Schröter (1897).

The genus *Pythium* is a member of the order Peronosporales of the class Oomycetes, the family Pythiaceae (Kirk et al, 2008). The genus *Pythium* was placed in Saprolegniaceae by Pringsheim (1858) and later in Peronosporaceae by de Bary in 1881 (Middleton, 1952). The genus *Pythium* is placed in the Pythiaceae family due to their undifferentiated sporangiophores, indistinct (conspicuous) periplasm, non-obligate parasites, or saprophytes (Ko et al, 2010). The morphological characteristics of the genus *Pythium* are hyaline or coenocytic hyphae without cross septa, sporangia filamentous or more or less spherical, asexual reproduction by means of zoosporangia and zoospores, zoospores formed in vesicle outside of sporangia, sexual reproduction through antheridia and oogonia, oospore covering the whole oogonium or leave some space between oogonia and oospore wall, undifferentiated hyphal swellings and appressoria club-shaped or sausage-shaped or sub-globe-shaped structure (Middleton, 1943; Plaats-Niterink, 1981).

Morphological characteristics such as the shape and size of sporangia or oogonia, antheridia per oogonium, the position of antheridium are the basis for differentiation of the species in the genus *Pythium* (Plaats-Niterink, 1981; Waterhouse 1963; Uzuhashi, Kakishima & Tojo, 2010). Around 85 *Pythium* species have been compiled by Plaats-Niterink (1981) in his monographs on *Pythium*. More than 150 species of *Pythium* have been described (Kirk et al, 2008). De Cock et al (2015) grouped species of *Pythium* into 14 clades ranging from A to K based on the molecular phylogenetic analysis. The intermediate species between *Phytophthora* and *Pythium* were grouped in *Phytopythium* (Bala et al, 2010). The advent of molecular approaches has added the potential to study the species in-depth, resulting in discovering more species, placing the species in different genera, or even creating new genera. Recently, Uzuhashi, Kakishima & Tojo (2010) started four new genera, Ovatisporangium, Globisporangium, Elongisporangium, and Pilasporangium, based on sporangial morphology and phylogenetic analysis and some species previously placed in the

genus *Pythium* have been moved to these new genera. There are currently 355 described species of *Pythium* based on mycobank (mycobank.org; Ho, 2018).

1.3.3 Phytophthora diseases

Phytophthora species are the most destructive pathogens of dicotyledonous plants, and a few of them are discussed in this section. *Phytophthora* species are extensively distributed worldwide from mild temperate to tropical regions. The *Phytophthora* species cause diseases in various hosts such as crops, forest trees, landscapes, ornamental plants, shrubs, and grasses (Zentmyer, 1983). Some of the most notable *Phytophthora* diseases are the late blight of potato, sudden oak death, taro leaf blight, damping-off and root rot of soybean, rhododendron root rot, citrus stem canker, and cocoa black pod. (Erwin & Ribeiro, 1996). *Phytophthora cinnamomi* causes root rot disease in more than 1,000 to 5,000 plant species (Jung, Colquhoun & Hardy, 2013; Gisi, Zentmyer & Klure, 1980). *Phytophthora cinnamomi* has caused massive devastation in Australian forests, causing root rot disease in more than 4,000 native species (Hardham & Blackman, 2018). The invasive *P. ramorum* causes sudden oak death in several plant species. *Phytophthora ramorum* has killed several million coastal live oak tan oak and significantly impacted the forest ecosystem in the USA, especially in California and Oregon (Grünwald, Goss & Press, 2008). *Phytophthora nicotianae* causes numerous diseases such as root rot, stem rot, foliage blight leaf spot, and stem canker disease on a wide range of hosts. *Phytophthora nicotianae* infects more than 300 plants, horticultural and vegetable crops, causing substantial economic loss (Erwin & Ribeiro, 1996).

1.3.3.1 Economic importance on agricultural crops

Phytophthora cinnamomi causes root rot disease in many crops. Some economically important crops affected by this pathogen are pineapple, chestnut, kiwi, avocado, etc. (Erwin & Ribeiro, 1996). *Phytophthora nicotianae* causes numerous diseases such as root rot, stem rot, foliage blight, leaf spot, stem canker, diseases in a wide range of hosts. *Phytophthora nicotianae* infects more than 300 species of plants, horticultural and vegetable crops causing substantial economic loss (Erwin & Ribeiro, 1996). *Phytophthora capsici* causes foliar and seedling blight, root, crown stem, and fruit rot of many agriculturally important vegetable crops such as pepper, cucurbits, onion, tomato, squash, and eggplant (Erwin & Ribeiro, 1996). *Phytophthora sojae* causes damping-off and root rot in soybean, causing an annual loss of $1

to 2 billion (Tyler, 2007). *Phytophthora palmivora* causes bud rot in coconut and oil palms. Recent epidemics of *P. palmivora* in Colombia have destroyed more than 70,000 ha of oil palm, causing significant economic loss (Torres et al, 2016). It has been estimated that *P. palmivora* has caused more than $250 million in losses from just two outbreaks in Colombia (Montoya et al, 2014). *Phytophthora infestans* infects all parts of its hosts and causes blight disease in solanaceous crops, mainly potatoes and tomatoes. *Phytophthora infestans* is widely distributed in potato production areas and causes huge economic loss every year. *Phytophthora erythroseptica* causes pink rot disease in potatoes, root rot in raspberry, and seed-piece of sugarcane (Erwin & Ribeiro, 1996). *Phytophthora erythroseptica* infects tubers in the field or storage causing significant losses.

The economic loss caused by most of these pathogens cannot be estimated in monetary form because of their wide range of hosts. These *Phytophthora* species cause substantial financial losses from yield loss, loss from disease management, seed, loss from storage rot, and in extreme cases, extinction of plant species in infested areas. Growers must modify their cropping systems if their fields are infested with certain species of *Phytophthora*. For example, *P. palmivora* causes disease in coconut and oil palms. Oil palm and coconut plantations must be avoided in the *P. palmivora* infested fields. Historically, *P. infestans* had a substantial socio-economic impact on the population of Ireland after the devastating Irish famine. Economic loss globally due to potato late blight from yield loss and the cost of disease management has been estimated to exceed $6.7 billion annually (Haverkort et al, 2008; Nowicki et al, 2012).

1.3.3.2 Epidemiology of diseases caused by *Phytophthora* species

Species in *Phytophthora* are found in aquatic sources such as streams, ponds, and terrestrial in a wide range of climatic conditions. The genus *Phytophthora* is very diverse and has a wide range of species. Since the *Phytophthora* species are polycyclic, they produce spores exponentially, and rapid disease progression occurs (Mackenzie et al, 1983). For example, late blight of potato and black pod of cocoa are the best examples of polycyclic *Phytophthora* disease (Erwin, Bartnicki-Garcia & Tsao, 1983).

The climate plays a vital role in adapting *Phytophthora* species to new sites. Some pathogenic species of Phytophthora in tropical climates might fail to be pathogenic in temperate climates and vice-versa (Erwin, Bartnicki-Garcia & Tsao, 1983). For example, cold

climates restrict the pathogenic activity of *P. cinnamomi* but do not restrict the pathogenic activity of *P. cactorum* (Erwin, Bartnicki-Garcia & Tsao, 1983). The micro-climate around the host plays a vital role in the infection, sporulation, and dispersal of *Phytophthora* species (Erwin, Bartnicki-Garcia & Tsao, 1983). The availability of water and low temperatures is directly related to the sporulation and release of zoospores in *Phytophthora* species (Erwin, Bartnicki-Garcia & Tsao, 1983). Temperature, leaf wetness period, and humidity or water status in the soil are major factors that play an important role in developing *Phytophthora* diseases (Erwin, Bartnicki-Garcia & Tsao, 1983). Species in *Phytophthora* produce oospores, sporangia, and zoospores, all of which can infect the host. Phytophthora propagules can also be airborne, soil-borne, or both.

The optimum temperature required for *P. infestans* to infect the host is 10 to 25°C with a high relative humidity of greater than 90% (Fry, Thurston & Stevenson, 2001; Narouei-Khandan et al, 2020). However, the soil temperature needs to be less than 18°C for *P. infestans* to infect tubers in saturated soil (Erwin, Bartnicki-Garcia & Tsao, 1983). The very wet soil and low temperatures assist the release of zoospores of *P. infestans*. Since the soil temperature is less variable, saturated soil for an extended period is conducive for the soil-borne *Phytophthora* species to infect root tissues (Erwin, Bartnicki-Garcia & Tsao, 1983). Soilborne *Phytophthora* species survive and release zoospores in very wet soil near saturation point. The zoospores first encyst and infect the host root tissue, then colonize vascular tissue and spread up the stem (Grünwald, Goss & Press, 2008). *Phytophthora ramorum* is primarily foliar and produces sporangia on an infected leaf or twig surfaces. It can be dispersed by air or water splash to the nearby hosts. *Phytophthora ramorum* can release zoospores in very wet soil, and zoospores can travel to nearby hosts, infect root hairs, colonize the vascular tissue, and move up the stem (Parke et al, 2007). Soil-borne species of *Phytophthora* are usually found in streams near infested areas and can be transported to new sites through irrigation or farm machinery.

In a broad range, the optimum temperature required for the *Phytophthora* species to infect the host and develop the disease is between 15-30°C (Erwin & Ribeiro, 1996). For example, the heart rot of pineapple (infected with *P. cinnamomi*) develops between 24 and 28°C, rhododendron root rot at 26°C, and root rot of avocado at 25°C (Erwin & Ribeiro, 1996). However, the optimum temperature for disease development for some temperate and

tropical *Phytophthora* species are in the lower and upper range, respectively (Erwin & Ribeiro, 1996). Some tropical *Phytophthora* diseases can occur at temperatures as high as 36°C and temperate *Phytophthora* diseases as low as 10°C (Erwin & Ribeiro, 1996).

1.3.3.2.1 Epidemiology of Phytophthora diseases on potato

Phytophthora erythroseptica was first described by Pethybridge in 1914 and was found to infect potatoes in Ireland (Erwin & Ribeiro, 1996). *Phytophthora erythroseptica*, the causal organism of pink rot, is a soil and seed-borne pathogen that poses a significant threat to potatoes in the field and storage (Lambert & Salas 2001; Powelson & Rowe, 2008). *Phytophthora erythroseptica* infects tubers in the field, at harvest, or during tuber handling operations and in storage. Infected tubers may be firm and rubbery with no symptoms, but infected tuber tissue turns pink when infected tubers are cut open and exposed to the air for 20-30 minutes (Lambert & Salas, 2001). The name "pink rot" was derived from this characteristic symptom (Wharton & Kirk, 2007a). However, infected tissue will eventually turn black after exposure to air for more than 30 minutes. Severely infected tubers may have blackened lenticels and buds, with white cottony growth on the tuber skin (Lambert & Salas, 2001). The initial symptomatic areas on the tuber are at or near the stolon end (Wharton & Kirk, 2007a). When tubers are cut open, the rotted portion of the tuber is delimited by a dark line that can be seen through the skin (Wharton & Kirk, 2007a). In the field, infected plants can show wilting, stunting, chlorosis, and yellowing symptoms on leaves, dark necrotic symptoms on stem and roots, and formation of aerial tubers (Lambert & Salas, 2001; Wharton & Kirk, 2007a).

The optimum temperature range for the growth of *P. erythroseptica* is 24-28°C, with 34°C being the upper limit (Lambert & Salas, 2001). Infected tubers may be severely rotted at 25°C. However, the pathogen develops rapidly at soil temperatures from 10°C to 30°C (Lambert & Salas, 2001; Wharton & Kirk, 2007a).

Phytophthora erythroseptica is found in most soils regardless of the crop grown and can survive for several years in the form of oospores in the soil or plant debris (Lambert & Salas, 2001; Wharton & Kirk, 2007a). The primary sources of inoculum for this pathogen are infected seed tubers, infected tubers in cull piles, volunteer tubers, or oospores in plant debris

or soil (Lambert & Salas, 2001; Wharton & Kirk, 2007a). The pathogen can infect the tubers at any stage of potato growth after tuber formation. *Phytophthora erythroseptica* can infect tubers through eyes, lenticels, the stolon end, and wounds and bruises (Lambert & Salas, 2001). The oospores formed on the volunteer tubers and tubers in cull piles are released into the soil after the tubers are entirely decayed (Wharton & Kirk, 2007a). The wet or waterlogged soil encourages oospores to germinate and sporangia to release zoospores during the spring or summer. Since zoospores are motile, they can swim in water films, encyst, and infect the progeny tubers (Wharton & Kirk, 2007a). If the soil is not waterlogged or wet, the oospore forms a germ tube and directly infects the underground stem, stolons, or roots (Wharton & Kirk, 2007a). If the tubers are infected in the field, healthy tubers can be contaminated during harvest and handling operations, causing disease in storage (Powelson & Rowe, 2008). During harvest and handling operations, wounds and bruises serve as entry points for the pathogen. Once infected tubers are stored, the infected tubers can transmit disease from one tuber to another in the storage (Wharton & Kirk, 2007a).

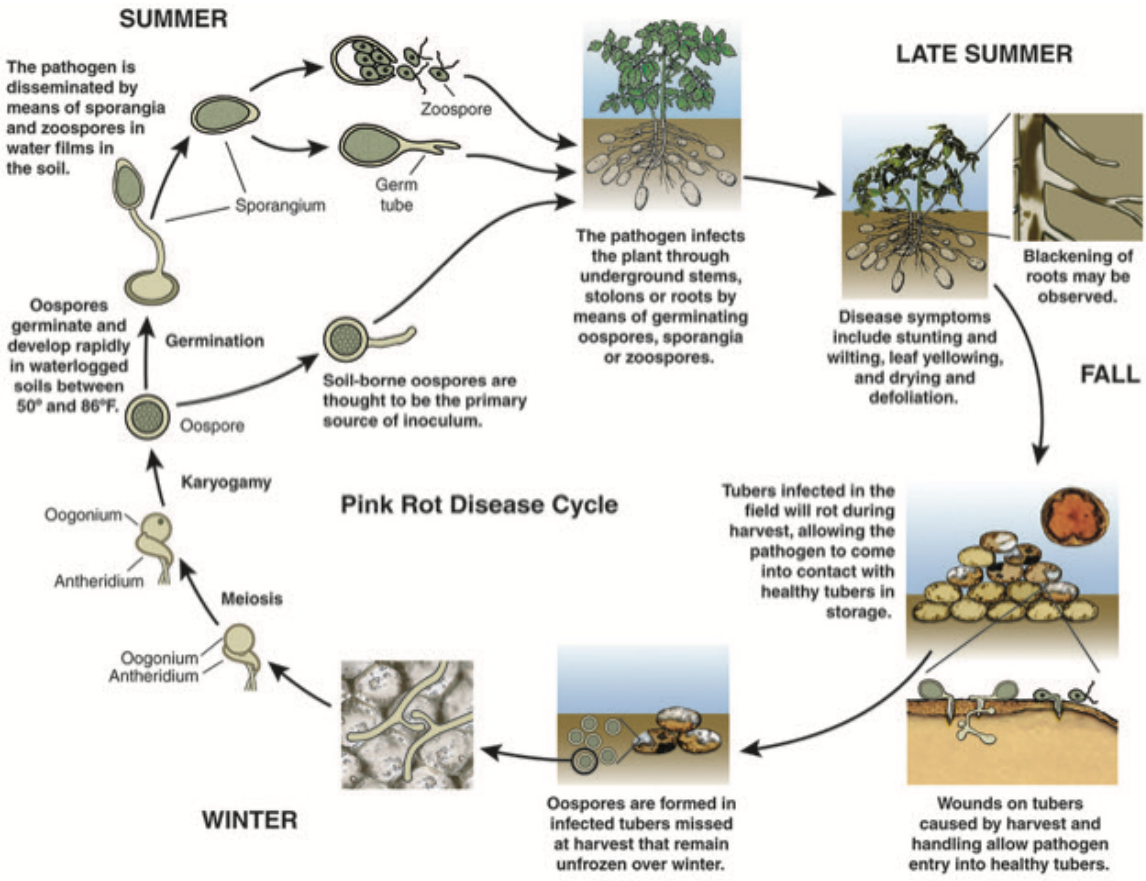

Figure 1.1 Disease cycle of the potato pink rot pathogen, *Phytophthora erythroseptica*, on potato. Image Courtesy Dr. Phillip Wharton.

Phytophthora infestans can infect all living tissue of the potato plant, so the symptoms of late blight can be seen on all parts of the potato. The symptoms of late blight begin with small necrotic lesions, which expand under conducive environmental conditions, and the surrounding tissue turns a pale green halo (Fry, Thurston & Stevenson, 2001). The pathogen is characterized by velvety, cottony growth of mycelia on the under-side of the leaf during cool, moist conditions, purplish discoloration of stems, and visible black spots and lesions on the tuber skin (Kirk et al, 2004). Severely infected plants become entirely defoliated, leaving only the dead brown stems. In tubers, internal symptoms of late blight are reddish to tan rust-like discoloration of tissue just below the surface. The skin remains intact, and the tuber is firm when squeezed (Kirk et al, 2004; Fry, Thurston & Stevenson, 2001). Usually, tubers with late blight in storage become contaminated with pectolytic bacteria like *Pectobacterium* spp., which rot the tubers completely (Wharton & Kirk, 2007b).

The optimal environmental conditions for *P. infestans* to produce sporangia and infect the potato plant are a moderate temperature between 10 to 25°C and high humidity (Fry, Thurston & Stevenson, 2001; Narouei-Khandan et al, 2020). At 18 to 24°C, sporangia cannot release motile zoospores, and instead, they form germ tubes to infect the tissue. However, this may vary with isolates of different genotypes (Fry, Thurston & Stevenson, 2001; Melhus, 1915; Mizubuti & Fry, 1998). Sporangia can also germinate and produce 8-12 biflagellate zoospores when there is free water available on leaf or tuber surfaces or in the soil when the temperature is between 4 and 12°C (Mizubuti & Fry, 1998) or 15°C (Melhus, 1915). Hyphae generated from sporangia or zoospores can penetrate potato tissue through lenticels, eyes, stomata, wounded or immature tissue (Lacey, 1967; Adams, 1975; Lapwood, 1977).

The primary sources of inoculum of this pathogen are infected seed pieces, volunteer potato tubers or plants, cull piles, and infected tomato seeds and plants (Johnson & Cummings, 2009; Kirk, 2003; Kirk et al, 2010; Zwankhuizen, Govers & Zadoks, 1998). Tubers intended for use as seed tubers are stored at 4ºC, and at this temperature, *P. infestans* remains in latent form (Johnson & Cummings, 2009). Thus, asymptomatic seed tubers are easily overlooked and contaminate other seed tubers during cutting and handling operations (Lambert, Currier & Olanya, 1998). Under conducive environmental conditions, mycelium from infected tubers infect emerging sprouts above-ground stems and sporulate during spring.

These sporulating sprouts can then infect healthy surrounding plants leading to the outbreak of an epidemic (Hirst & Stedman, 1960; Johnson, 2010; Kirk et al, 2009). The pathogen has a short secondary asexual cycle of 4 - 7 days, producing millions of sporangia exponentially (Kamoun & Smart, 2005). This results in the rapid spread of the pathogen to other healthy leaves and stems under cool and moist weather conditions (Kirk et al, 2004). Infection of susceptible tissue occurs for as few as 2 hours (Fry, Thurston & Stevenson, 2001). Any irrigation or rainfall late in the growing season can spread the sporangia and zoospores down into the soil resulting in the infection of progeny tubers (Fry & Goodwin, 1997a; Hirst et al, 1965; Lapwood, 1963). Harvesting tubers when the vines are still green or when the skin of the tubers is not well-formed makes tuber infection even worse and may result in severe tuber rot in the storage (Bonde & Schultz, 1945; Miller et al, 2002). Infected seed tubers are also exported to different countries around the globe, contributing to the migration and introduction of new isolates of the pathogen to new places. Thus, any infected seed or table stock tubers are a severe problem in managing late blight.

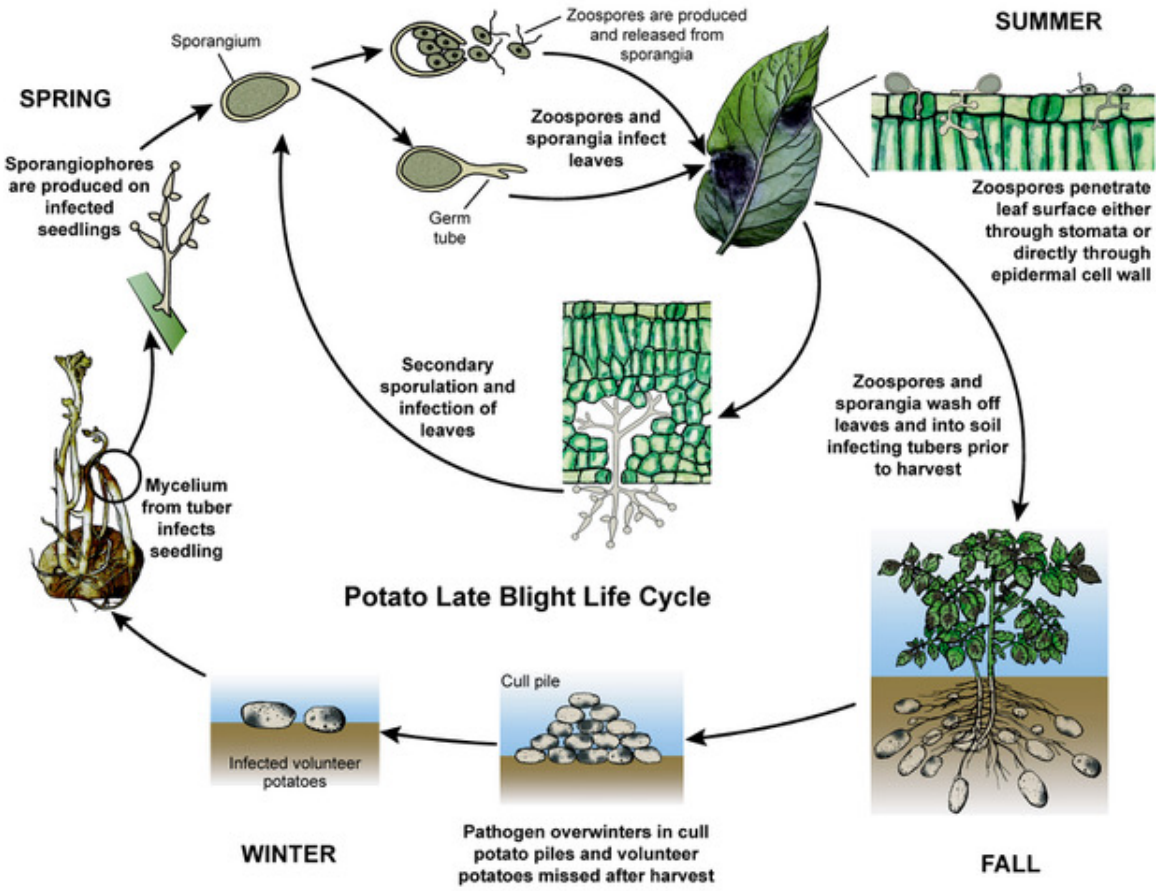

Figure 1.2 Disease cycle of the potato late blight pathogen, *Phytophthora infestans*, on potato. Image courtesy Dr. Phillip Wharton.

1.3.4 Pythium diseases

Pythium species are usually saprophytes and infect mainly monocotyledons. The *Pythium* genus causes several diseases and significantly affects world agricultural production and forest ecosystems. *Pythium* species infect mostly juvenile and succulent plants or seedlings. *Pythium* causes pre- or post-damping off of seed and seedlings, fruit rot, stem rot, and root rot of many plants (Plaats-Niterink, 1981). *Pythium aphanidermatum* is one of the most destructive *Pythium* species and causes root rot, damping-off, stalk rot, rhizome rot, fruit rot, and many more in a wide range of hosts (Plaats-Niterink, 1981). *Pythium aphanidermatum* is found in many countries, especially warm climatic regions (30-35°C). *Pythium graminicola* was first reported on wheat. *Pythium graminicola* causes root rot on several species of the Gramineae family, including maize, sorghum, zinger, creeping bentgrass, and is distributed in many countries (Craft & Nelson, 1996; Plaats-Niterink, 1981). *Pythium ultimum* infects many plants causing damping-off and foot-rots, stunting and damping-off of soybeans, wheat, sugarcane, peach, potato, citrus poinsettia (Plaats-Niterink, 1981). *Pythium ultimum* is widespread in many countries around the globe. *Pythium vexan* causes many diseases in several plants and crops, including potato, sweet potato, ornamentals, carnation, pecan seedlings. *Pythium vexan* causes patch canker in Hevea. It was also found together with *P. cinnamomi* in declining peach trees (Craft & Nelson, 1996; Plaats-Niterink, 1981). *Pythium irregulare* causes potato rot, damping-off, and root rot in many cereal, legume, fruit, and vegetable crops. It infects more than 200 species of plants (Plaats-Niterink, 1981).

1.3.4.1 Economic importance on agricultural crops

Pythium causes damping-off diseases of many economically important vegetable, field, ornamental, and horticultural crops. The *Pythium* genus has a wide host range causing diseases in several economic crops. *Pythium* species such as *P. aphanidermatum*, *P. irregulare*, and *P. ultimum* cause seed rot, root rot, seedling rot, seedling damping, and blackleg of many ornamental and floriculture crops. These diseases cause a substantial economic loss in the floriculture industry (Del Castillo Munera & Hausbeck, 2016). In the United States, the seedling damping-off of soybean was the second most destructive disease caused by *Pythium* species along with other seedling rot pathogens and caused severe yield

loss in the soybean crop (Allen et al, 2017). In many vegetable crops, *P. aphanidermatum*, *P. irregulare*, and *P. ultimum* cause significant damage to the germinating or emerging seedlings killing them outright or resulting in poor emergence. In the post-damping off, the pathogen girdles the crown, infects the root and stem, kills the whole plant, or weakens the plant stand. In the worst-case scenario, growers need to re-plant the entire field. *Pythium* leak caused by *Pythium ultimum* is another economically important disease of potato. Potatoes infected with *Pythium ultimum* rot in the field or later in the storage, causing severe yield and economic loss.

1.3.4.2 Epidemiology of diseases caused by *Pythium* species

Pythium species are cosmopolitan with a wide host range and distributed from tropics to sub-temperate climates (Middleton, 1943; Plaats-Niterink, 1981). *Pythium* species are found in cultivated or fallow soils, fresh or pond water, plants, and some aquatic animals (Parveen & Sharma, 2015). Temperature and water availability are the major factors for *Pythium* disease development. Since *Pythium* species are soil-borne or water-borne, they need saturated soil to release zoospores (Plaats-Niterink, 1981). Under conducive environment conditions for *Pythium* species and unfavorable conditions for the host, they can be very pathogenic (Plaats-Niterink, 1981). The infection of the host by *Pythium* species depends upon many factors such as inoculum density, soil water availability, temperature, pH, light, light intensity, and other microflora (Plaats-Niterink, 1981). The pathogenic intensity of *Pythium* species also depends on the availability of pectolytic and cellulolytic enzymes (Plaats-Niterink, 1981). Since species of *Pythium* have different levels of these enzymes, some can be very pathogenic and some less or non-pathogenic. The optimum temperature for *Pythium* species to grow has a wide range, but maximum species grow and develop diseases at 28 to 37ºC. A few *Pythium* species grow and develop the disease as low as 1ºC and as high as 43ºC (Middleton, 1943; Plaats-Niterink, 1981). For example, *P. aphanidermatum* infects cucurbits severely at 30 to 35ºC, *P. myriotylum* infects and cause pod rot of peanut at 30ºC, *P. graminicola* infects cereals at 13-28ºC, and *P. ultimum* infects host severely at 23ºC (Gay, 1969; Nelson & Craft, 1991; Thompson, Athow & Laviolette, 1971).

1.3.4.2.1 Epidemiology of *Pythium* diseases on potato

Pythium ultimum var. *ultimum* Trow was initially isolated from rotten cress seedlings in England by Trow in 1901 (Plaats-Niterink, 1981). *Pythium ultimum,* the causal agent of Pythium leak, can cause disease in the field and storage. Pythium leak is one of the most important storage rots as it causes significant storage loss (Salas & Secor, 2001). The disease is called *Pythium* leak, watery rot, or shell rot because of the nature by which it rots the tuber leaving the periderm or skin intact while rotting out the center of the tuber (Powelson & Rowe, 2008; Salas & Secor, 2001). Tubers infected with *Pythium* remain firm, but if cut in half and then squeezed, there is a clear watery discharge from the tuber. The name "*Pythium* leak" was derived from this characteristic symptom. The pathogen can infect the tubers at any stage of potato production (Salas & Secor, 2001). Typical symptoms of infected tubers are well-defined silvery lesions or water-soaked lesions, discoloration of tuber skins with internal tissue being smoky gray to black with a distinct dark line between the healthy and infected tissue (Johnson, 2020; Salas & Secor, 2001).

Pythium leak is more severe at high temperatures, high humidity, and in wet soils (Lui & Kushalappa, 2003). The optimum range of temperature for *Pythium ultimum* to cause disease is 15 to 30°C, and disease generally does not develop below 10°C (Salas & Secor, 2001). The infected tubers start showing symptoms at 18°C, which are severe above 25°C (Johnson, 2020). At higher temperatures, the symptoms are visible in as few as 36 hours (Johnson, 2020).

Pythium ultimum is a ubiquitous soil-borne pathogen found in the form of oospores, in plant debris, or infecting weeds in all potato growing areas (Salas & Secor, 2001; Secor & Gudmestad, 1999). The initial infection can start in the field right after planting, during the growing season before or at harvest, during handling operations going into storage, or in storage (Salas et al, 2003; Salas & Secor, 2001). Mycelia, germinating sporangia, or oospores of *P. ultimum* are all infectious. These propagules can enter the tuber tissue through cuts, bruises, and wounds on the seed piece before tuber suberization occurs (Powelson and Rowe, 2008; Salas and Secor, 2001). The disease is more severe when the soil is too wet and high temperature at harvest (Lui & Kushalappa, 2003). Unlike *P. erythroseptica* and *P. infestans*, *P. ultimum* cannot infect tubers through the eyes. Rarely, *Pythium* infects the tuber through

lenticels and stem buds (Johnson, 2020). Usually, the pathogen infects tubers through bruises and wounds during harvest as it cannot penetrate the intact periderm of the tuber (Taylor, Pasche & Gudmestad, 2008). Once the pathogen gets established in the tuber tissue, the rate of tissue disintegration increases with conducive environmental conditions such as high humidity or wetness and high temperature (Lui & Kushalappa, 2003). Once infected tubers are stored, the infected tuber can transmit disease from one tuber to another. Under optimal conditions, *Pythium* is a fast-growing pathogen. It often acts as a gateway for secondary infection of tubers by *Pectobacterium* spp, resulting in soft rot in the storage (Wharton & Kirk, 2007b). In such cases, the whole tuber can be reduced to mush in a matter of days. The entire storage "melts down" as the entire pile of tubers is decayed to liquid by soft rot. Usually, the oospores resting in the soil serve as a primary source of inoculum (Erwin & Ribeiro, 1996).

1.4 Management of oomycete pathogens

Late blight, Pythium leak, and pink rot diseases are the major concern in many potato production areas around the globe. The timely, accurate, and rapid identification of pathogens allows growers enough time to make proper decisions on best management strategies (De Boer & Lopez, 2012; DeShields et al, 2018). An integrated management approach is required to manage these diseases effectively and sustainably. An integrated system needs to include cultural, biological, chemical, and resistance cultivar approaches. The type of integrated approach to control *P. infestans*, *P. erythroseptica,* and *P. ultimum* dependents on the isolates or genotypes present in the fields because different isolates or genotypes have a different response to mefenoxam fungicide (Taylor et al, 2002; Taylor, Salas & Gudmestad, 2004; Danies et al, 2013; Goodwin, Sujkowski & Fry, 1996).

1.4.1 Cultural practices

The main aim of cultural practices is to exclude or avoid any form of disease organisms. Exclusion or avoidance may be done by decreasing inoculum levels, limiting reproduction, dispersal, and contact of the plant with the pathogen in the field and storage. The primary source of inoculum for the initiation of late blight epidemics are any form of infected tubers such as seed tubers, volunteers, cull piles, and rarely oospores in the soil (Bonde & Schultz, 1943; Zwankhuizen, Govers & Zadoks, 1998; Kirk, 2003; Fernández-

Pavía et al, 2004; Johnson & Cummings, 2009; Kirk et al, 2010). Hence, the cultural management of *P. infestans* includes using the certified seed, removing volunteer tubers plants, and proper disposal of cull piles (Majeed et al, 2014; Secor & Gudmestad 1999; Zwankhuizen, Govers & Zadoks, 1998). Since high humidity or moisture increases the risk of disease development, the leaf wetness period should be reduced by appropriately timing the irrigation (Fry, Apple & Bruhn, 1983). The part of the field likely to get more water or waterlogged conditions should be scouted regularly to check for any disease development (Kirk et al, 2004). Harvesting of tubers when the vines are still green or when the skin of the tubers are not well set increases the potential of tuber infection (Bonde & Schultz, 1945; Miller et al, 2002). So, harvesting should be done 2-3 weeks after vine kill.

Field selection with well-draining soil, crop rotation with non-host crops, limiting irrigation towards the end of the growing season before harvest, giving tubers enough time to set skin after vine kill, and harvesting tubers during cool, dry conditions are crucial for the cultural management of oomycete diseases (Lambert & Salas, 2001; Powelson & Rowe, 2008). Oomycete pathogen thrives well in waterlogged conditions; hence over-irrigation should be avoided (Wharton et al, 2009). Harvesting tubers when the pulp or pre-harvest temperature is below 15°C and maintaining storage temperature and humidity helps reduce the potential of storage rot from these oomycete pathogens (Wharton et al, 2009). The suberization process should be enhanced by lowering the temperature to 15°C and humidity greater than 90% (Salas & Secor, 2001).

1.4.2 Chemical control

Since it might not be possible to control oomycete disease with cultural practices, fungicides are essential to combat these diseases. Fungicides are used as seed treatments, in-furrow, or foliar treatments to protect the seed and plant right from the very beginning of crop production. Both protective (applied before the infection) and curative (applied when symptoms are seen) fungicides are available to control oomycete diseases. Protective fungicides should be used before infection or germination of the pathogen on seed pieces to suppress the pathogens (Bruck et al, 1981).

Fungicides in the group of Phenylamides (PA), the quinone outside inhibitors (QoI), carboxylic acid amides (CAA), and multisite inhibitors (M) have been used to manage

oomycete diseases (FRAC; Gisi & Sierotzki, 2015). However, sometimes these fungicides are mixed with other fungicides to have additive effects. Metalaxyl is a systemic fungicide in the Phenylamide group. Metalaxyl has been used to control oomycete disease for a long time (both protective and curative, Goodwin, Sujkowski & Fry, 1996; Torres, Martin & Henfling, 1985). Non-systemic fungicides such as mancozeb and chlorothalonil are applied well before the appearance of any symptoms because of their inhibitory effect on spore germination in the plant tissue (Baibakova et al, 2019; Bruck et al, 1981; Cohen & Coffey, 1986). However, systemic fungicides such as metalaxyl can be used both before and after the infection because these fungicides are absorbed in plant tissue and transported to the different parts of the plant to prevent infection or inhibit the expansion of infection (Baibakova et al, 2019; Bruck, Fry, and Apple, 1980; Cohen & Coffey, 1986; Oliver and Hewitt, 2014;). Fungicides such as fenamidone (QoI), mancozeb (M3), chlorothalonil (M5), cymoxanil (cyanoacetamideoximes), mandipropamid (CAA), flutolanil (carboxamides) + mancozeb (M3), fludioxonil (PP) + mancozeb (M3) are reported to be very effective in controlling late blight, pink rot, and Pythium leak (Powelson & Inglis 1999, Rekanovic et al, 2010, Stevenson, 2008; Wharton & Kirk, 2007a, b). Still, these fungicides should be used in the recommended dose and before the infection.

Many fungicides are registered for foliar sprays to control foliar disease in the field; however, there are very few fungicides or chemical options available to control storage diseases. Some disinfectants such as chlorine dioxide, hydrogen peroxide, and peroxyacetic acid have been used in low doses before the tubers are stored in storage but were partially effective (Olsen et al, 2003; Miller et al, 2006). These disinfectants can kill only surface pathogens. However, these disinfectants cannot protect the tubers that are already infected in the field or during harvest. Metalaxyl and phosphoric acid have been used for a long time and are more effective when used as foliar sprays at the end of the season (Johnson, 2008; Johnson et al, 2004; Miller et al 2006). The metalaxyl resistant isolates of *P. infestans*, *P. erythroseptica,* and *P. ultimum* have evolved, making this fungicide ineffective (Goodwin et al, 1996; Taylor et al, 2002). However, metalaxyl can still be used in those locations where resistant isolates of the oomycete pathogens have never been found. In some experiments, Phosphorous acid sprays were effective post-harvest pre-storage treatment for tuber blight and pink rot but were ineffective to control Pythium leak (Gachango et al, 2012; Miller et al,

2006; Johnson et al, 2004). The other alternatives to fungicides are plant volatiles with some promising results on some fungal diseases but limited control on oomycete diseases (Wood et al, 2013). Other systemic foliar fungicides are being evaluated for in-season application and can be used to protect the tubers in storage from storage rot pathogens.

Fungicide resistance is a widespread problem in most potato pathogens, including oomycete pathogens. The outbreak of metalaxyl resistance genotypes (US-8) of *P. infestans* in the 1990s had caused substantial economic loss (Goodwin et al, 1998; Goodwin, Sujkowski & Fry, 1996). In recent years, *P. ultimum*, *P. erythroseptica,* and *P. infestans* resistance to mefenoxam have been reported (Matson et al, 2015; Taylor et al, 2002). Therefore, metalaxyl should not be used in those potato production sites where resistant isolates have been reported. Since the new isolates of *P. infestans* are becoming resistant to new fungicides such as fluazinam, fungicides should be rotated or tank-mixed to avoid or manage resistance or increase the life of fungicides according to FRAC (Schepers et al, 2018).

1.4.3 Resistant cultivars

Disease-resistant cultivars of potatoes significantly impact integrated management because of their durable long-term effect and environment friendly. Resistant cultivars considerably reduce the need for fungicide sprays. No cultivars are immune to oomycete pathogens and most commercial cultivars currently available are susceptible (Kirk et al, 2004). However, some cultivars are less susceptible than others, such as Atlantic and Snowden are less susceptible to pink rot and Pythium leak (Salas et al, 2003; Thompson et al, 2007).

Frequent expensive systemic fungicides are required to combat late blight in high disease pressure locations (Kromann et al, 2009). So, using resistant cultivars in such areas would be more beneficial. Some late blight-resistant cultivars available in the USA are Defender, Jacquelin Lee, Missaukee, Tollocan, and Torridon (Douches et al, 2001; 2004; 2010; Novy et al, 2006). However, these cultivars were developed in reference to the US or known European genotypes of *P. infestans,* so they might not be resistant in other locations with different pathogen strains.

The potato is thought to have originated from the Andes region of South America; many wild *Solanum* species were found to have resistance genes to late blight in these regions

(Bradshaw & Ramsay, 2009; Hawkes & Hawkes, 1990; Hawkes & Francisco-Ortega, 1993; Jansky, 2000). Scientists have utilized these wild germplasms from wild species of Solanum to develop resistant cultivars. In the 20th century, resistant cultivars were developed through a single *R* gene (*R1*, *R2*, *R3,* and R*10*) from wild *Solanum demissum* using conventional breeding techniques (Park et al, 2009; Umaerus, 1994). The cultivars with a single *R* gene from *Solanum demissum* conferred resistance in the field with few tester genotypes of *P. infestans* for few years (Fry, 2008). However, due to selection pressure, the low population of *R* gene-breaking strains/genotypes of *P. infestans* were evolved (Fry, 2008). Such cultivars with one *R*-gene were quickly overcome by new genotypes or clonal lineages of *P. infestans* (Umaerus, 1994). Such new clonal lineages are the descendant of parental genotypes that arise mainly through mutation and mitotic recombination (Goodwin, Cohen, & Fry, 1994; Danies et al, 2013). The *P. infestans* population becomes more diverse if new genotypes are introduced through seed tubers in recent locations. In such a scenario, stacking multiple genes is prudent to protect the crop from the new genotypes of *P. infestans*.

After the failure of single *R* gene resistant cultivars, scientists came up with the idea of stacking multiple *R* genes to maintain the efficacy of *R* genes (Fry, 2008). Subsequently, a 3-*R* gene (*R1*, *R2*, and *R3*) cultivar resistant to the Race 4 strain of *P. infestans* was developed in Scotland. However, the *R* genes of cultivars were overcome by a new genotype of *P. infestans* which evolved in few years after the cultivar was released (Fry, 2008; Malcolmson, 1969). Conventional breeding takes 10-15 years to develop late blight resistant cultivars with multiple *R* genes, and by then, the population of *P. infestans* might change, and the resistant cultivars might be no longer resistant to *P. infestans*. Less time-consuming bioengineer or genetic modification (GM) technology has been developed to clone the resistant genes from different species of *Solanum* (Park et al, 2009). Multiple genes from other *Solanum* species can be introgressed in the potato cultivars with a bioengineer or GM approach. The potato cultivars with bioengineered 3-R genes from different *Solanum* species are more durable and have no additive genes from the wild *Solanum* species that alter the different qualities of potatoes. Since the late blight-resistant cultivars commercially available might not be the choice of consumers, in such a scenario stacking 3-R genes in the consumer-preferred cultivars would be an alternative. Recently, local cultivars “Desiree” and “Victoria” were developed using a bioengineering or GM approach with 3-*R* genes resistant to late blight

(Ghislain et al, 2019).

Resistant cultivars might have some issues during the adoption process. The first issue is that the newly developed cultivars may not provide resistance against late blight because the genetic makeup of the late blight isolates present locally might be different. The second issue is that growers can be reluctant to adopt new cultivars because new cultivars might differ in taste and are less preferred by the consumers. The third issue, the local soil type and micro-climate, may not be suitable so that the potential yield might be compromised. These issues should be considered while developing and releasing the new cultivar in a new location. Even after resistant cultivars are released, fungicide management programs should be maintained for durable resistance. A reduced rate of fungicides can be applied to the resistant cultivar, as reported by Kirk et al (2001) and Ghislain et al (2019).

1.4.4 Monitoring and disease forecasting

Early detection of potato disease visually or using molecular approach is crucial for successful disease management. Early detection helps control the disease in an early stage to minimize the loss and reduce the cost of disease management. Early warning or detection of disease also gives growers more time to plan disease management strategies and apply the right disease management tool at the right time. There are several disease forecasting models to forecast late blight. The main forecasting systems will be discussed in this section.

1.4.4.1 Disease scouting

Potato fields should be regularly scouted after crop emergence for any signs and symptoms of diseases. Phytotoxic or chemical and mechanical injuries can be confused with disease symptoms because phytotoxic or chemical injured plants show symptoms like burned leaf lesions, necrotic patches, wilting, and damping-off (Korus et al, 2015). The patterns of symptom distribution in the field and on plant parts can give a better understanding of the plant symptoms, whether these are due to chemical injuries or disease (Korus et al, 2015). However, samples with confusing symptoms should be sent to a diagnostic lab for correct identification. Scouting should be primarily focused on those parts of the field that may have been missed by fungicide sprays, waterlogged spots, parts of the field shaded by windbreaks, and low spots with high moisture (Kirk et al, 2004). If the irrigation is through a central pivot system, then the central or focal point which gets more water should be scouted with extra

care. During cool and high humid conditions, the lower canopy of the potato crop should be carefully checked. Sanitation should be maintained while scouting from one field to another since the spores of pathogens can be easily transported on clothing and equipment.

1.4.4.2 Disease forecasting

Disease forecasting gives the grower information about the likeliness or unlikeliness of disease occurrence based on the environmental conditions. Disease forecasting helps to save the expenditure of time, money, and labor, thus helping to cut down the cost of production. Disease forecasting programs predict disease based on favorable weather conditions, mainly relative humidity (RH) and temperature, suggesting that the growers start fungicide sprays (Kirk et al, 2004). There are several disease forecasting models to forecast late blight. The primary forecasting system will be discussed in this section.

Blitecast is one of the oldest computerized late blight forecasting systems used to schedule fungicide applications based on a modification of two late blight forecasting systems, Hyre's and Wallin's systems (Krause et al, 1975). The major component of this forecasting system is rainfall, relative humidity (RH), and temperature. Hyre's system is based on daily rainfall records and maximum and minimum temperatures (Krause et al, 1975). Wallin's system forecasts the initial occurrence and subsequent spread of late blight based on RH and temperature (Krause et al, 1975). Wallin's system is based on the seasonal accumulation of "severity values." Disease severity values (DSV) rely on a specific relationship between the number of hours of specified RH threshold (RH >90%) and the average air temperature during those periods (Krause et al, 1975). Any DSV value greater than 18 means fungicide application should be started (Krause et al, 1975). Blitecast suggests fungicide sprays when the severity and rainfall value reach 1 (7-day interval spray), 2 (5-day interval spray), 0 late blight warning, and -1 No spray. (Krause et al, 1975).

BlightPro decision support system (DSS) for late blight of potato and tomato was developed based on weather conditions, crop information, and management tactics. Decision support systems are tools as simple as data processing or as complex as a computerized expert system to support extension agents, consultants, and growers in crop production management, including plant pathogens (Magarey et al, 2002). The BlightPro DSS links several models into the system (Small, Joseph & Fry, 2015). The recommendation for foliar applications to

the specific location is based on weather data, crop management information to drive the disease forecasting systems, and a validated mechanistic disease model (Small, Joseph & Fry, 2015).

Smith's forecasting depends on two-consecutive 24h periods in which the minimum temperature is not below 10°C, and there are at least 11 hours each day with RH >90% (Smith, 1956). This forecasting system was used for 60 years without revision or modification. Smith's forecasting model was average since there was significant variation in climate within Great Britain (Dancey, Skelsey & Cooke, 2017). According to Smith's model, if late blight favorable conditions have occurred in an area, the forecasting system indicates the possibility of primary infections and production of spores of *P. infestans*. Based on this speculation, spray programs are recommended. However, this is an overestimation. Smith's criteria were compared with lowering the RH value from 11h to 6h in nine defined climatic regions of the UK (Dancey, Skelsey & Cooke, 2017). There was a significant increase in the accuracy of the alert system when RH was set to 80% instead of 90%, and it more accurately predicted the late blight risk. Hutton criteria were developed to forecast late blight by modifying the RH period from 11h to 6h (Dancey, Skelsey & Cooke, 2017). The Hutton Criteria has recently been used in Great Britain to identify the high-risk periods for disease development (Dancey, Skelsey & Cooke, 2017).

The Resilient propagation (Rprop) neural model was the most effective late blight model based on comparison with risk estimated with Unedited Local Climatological Data (ULCD) for late blight prediction in Michigan, USA (Baker & Kirk, 2007). The Rprop model was effective, especially between June and August, the critical time for late blight occurrence. The Rprop model is based on modified-Wallin's disease severity model. Another web-based expert system was developed based on modified Wallin-type model. The relative humidity (80%) and temperature from a network of automated weather stations in Michigan was used to predict the favorable environment conditions for late blight development (Wharton et al, 2007). The management recommendations are produced for each location based on the risk level. The expert system is web based and interactive allowing growers to access site specific weather-based recommendations through personal computers (Wharton et al, 2008). In addition to that, this system includes data from a network of regional weather stations in daily

risk estimates and management recommendation for 48 locations in Michigan (Wharton et al, 2008).

1.5 Techniques for detection and characterization of oomycete pathogens

Traditional diagnostic methods include direct plating, culture, and serial dilution of soil or infected plant tissue. Baiting has been used to collect samples from different sources. Morphological characteristics of culture were the key to the identification of pathogens. Serial dilution, either using bait or the soil, has also been used to identify potato pathogens (Larkin, 2008; Steere et al, 2015; Pasche et al, 2013). These traditional methods are time-consuming and need to be carried out by a well-trained person.

Nowadays, numerous diagnostic assays are based on molecular markers to detect pathogens in potato crops. In many studies, the enzyme-linked immunosorbent assay (ELISA) has been used to diagnose potato pathogens such as viruses, fungal, oomycete, and bacterial pathogens. ELISA assays are the diagnostic tools to detect specific antibodies, soluble antigens, or cell surface antigens (Hornbeck, 1992). In ELISA assays, solid-phase reactants can be antigen or antibody adsorbed onto the plastic microtiter plates or cell-associated molecules (Hornbeck, 1992). The specific antigen in the samples only binds to its antibody/associated molecule or vice-versa (Aydin, 2015). In brief, the soluble reactant/enzyme-linked conjugate binds specific solid-phase reactant/enzyme-linked substrate on a microtiter plate (Aydin, 2015). The unbound enzyme conjugates are washed out, and the chromogenic or fluorogenic substrate is added to hydrolyze the bound enzyme conjugates (Hornbeck, 1992). The results are read visually or using a microtiter plate reader (Hornbeck, 1992). More than 10 million tests detect agricultural pathogens every year using ELISA (Bonants et al, 2005).

Lateral flow immunoassays (LFIAs) are rapid on-site diagnostic tools to detect and quantify the analyte based on antigen and antibody. In LFIAs, antibodies are exclusively used as recognition elements (Koczula & Gallotta, 2016). These cost-effective and on-site detection kits are designed for single use. The LFIA has different zones; the analyte to be tested is loaded on the adsorbent sample pad zone. Due to the capillary force provided by the adsorbent zone on the other end, the liquid sample migrates to conjugate release, detection,

control line, and adsorbent pad zone (Koczula & Gallotta, 2016). In each zone, a specific reaction occurs, and the sample is detected positive or negative based on the specific antigen in the target analyte. The conjugate pad zone contains antibodies specific to the target analyte, whereas the detection zone contains antibodies or antigens that recognize the conjugated antibody (Koczula & Gallotta, 2016). The test line responds to appropriate recognition by the detection zone, and the control line confirms the proper migration of liquid (Koczula & Gallotta, 2016). Recently, many LFIA assays have been developed and used to detect potato bacteria, viruses, oomycetes in the field (Panferov et al, 2016, 2018; Safenkova et al, 2016; Zhan et al, 2018).

Polymerase Chain Reaction (PCR) is a molecular technique to amplify a small piece of DNA using a pair of oligonucleotides, thermostable *Taq* DNA polymerase, and suitable master mix buffer (Erlich, 1989). The mixture of reagents and DNA samples are then run for several hours in a repetitive heating and cooling cycles in a thermocycler until the desired amount of amplification is achieved (Erlich, 1989). Generally, the heating cycle at high temperature 95°C is desired to denature the DNA in single strands, cooling cycle at around 60°C for primers to anneal to each original strands and extension of the newly formed DNA strands at 70°C. The PCR technique became popular to study different gene of interest because of its simplicity and high probability (Erlich, 1989). Later, more advanced form of PCR such as quantitative PCR (qPCR) and reverse-transcriptase PCR (RT-PCR) were developed to detect and quantify the target DNA. Quantitative PCR (qPCR) is a molecular approach for detecting and quantifying the target DNA of pathogens or any organisms. qPCR uses a pair of species-specific oligonucleotides with or without fluorescence labelled probe, thermostable *Taq* DNA polymerase, and suitable master mix buffer and run in a thermocycler capable to read the fluorescence generated from the reaction. PCR and qPCR are the most widely used gold standard techniques used in many diagnostic laboratories. Several PCR, qPCR, and reverse transcription PCR assays have been developed to detect and identify potato pathogens. RT-PCR is used to identify different strains and types of viruses (Karasev et al, 2008). Real-time quantitative PCR assays have been developed for the detection of potato storage rot pathogens using the ITS region of pathogen DNA (Cullen et al, 2007). These assays could reliably differentiate *Pythium ultimum*, *P. erythroseptica* from other watery wound rot causing potato pathogens. The potato pathogens can be detected and identified

from different sources, such as soil and plant parts, using different qPCR assays (Brierley et al, 2009; Cullen et al, 2000, 2001, 2002; Lees et al, 2002).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification technique that can be run under isothermal conditions and eliminates the odds of PCR and other molecular techniques (Abdullahi et al, 2015; Zhang, Lowe & Gooding, 2014). Unlike conventional PCR, the LAMP technique does not require heating and cooling steps because the *Bst* DNA polymerase in the LAMP assays has DNA strand displacement activity under isothermal conditions (Notomi et al, 2000; Zhang, Lowe & Gooding, 2014). LAMP assay requires a set of four to six specifically designed primers. These four to six primers can recognize six distinct regions of the target simultaneously, hence amplifying the target DNA with high efficiency and precision (Notomi et al, 2000). Therefore, the LAMP technique is fast, accurate, and robust than other DNA amplification techniques. LAMP assays have higher sensitivity and specificity than PCR-based assays. LAMP's simplicity, low running cost, robustness, sensitivity, and accuracy have a huge advantage over traditional molecular methods (Parida et al, 2008). LAMP technology has shown promising results for diagnosing plant pathogens on multiple crops (Bühlmann et al, 2013; Tomlinson, Dickinson & Boonham, 2010b; Tsui et al, 2011). LAMP assays based on *dnaA* and 16s rRNA gene regions have been developed to detect the bacterial pathogen causing potato blackleg (Ocenar et al, 2019). Sagcan & Kara (2019) developed another assay using the 16s rRNA gene region to detect *Clavibacter michiganensis* in potatoes. These LAMP assays are accurate and reliable due to their high sensitivity and specificity. Also, the LAMP assays are reported to be less sensitive to inhibitors, unlike PCR assays (Francois et al, 2011).

Sequencing is another molecular approach to identifying the plant pathogen and is used for phylogenetic analysis. Sequencing does not need previous knowledge of the pathogen. Generally, internal transcribed regions (ITS) of ribosomal DNA are sequenced because they are highly conserved and taken as a universal barcode of fungus (Peay, Kennedy & Bruns, 2008; Schoch et al, 2012). Other regions targeted for a sequence are cytochrome oxidase (cox 1 and cox 2) spacer regions, nuclear translation elongation factor 1α- and β-tubulin gene (Kroon et al, 2004; Villa et al, 2006; Blair et al, 2008; Robideau et al, 2011).

1.5.1 Conventional methods of oomycete identification

Baiting techniques have been used for many years and are still used to identify many oomycetes species collected from aquatic and terrestrial sources (Werres et al, 2014). The host materials are used to bait the pathogens from a water source or field. For example, Rhododendron leaves have been used to bait *P. ramorum* in streams in agricultural and forest areas (Shrestha et al, 2013). Several studies have used cucumber, squash, or pears in ponds and ditches near agricultural lands and soil to bait *Phytophthora* spp (Erwin et al, 1983; Gevens et al, 2007). Leaves or fruits used as bait were incubated, and any symptomatic lesions were transferred to selective media (Erwin et al, 1983; Gevens et al, 2007; Shrestha et al, 2013). The genus and species of recovered isolates were identified based on morphological characteristics using microscopy and taxonomic identification keys. Currently, recovered cultures can be sequenced to distinguish the species of *Phytophthora* based on phylogenetic analysis.

In the past, plant-pathogen used to be diagnosed solely through observation of morphological structures under the microscope or growth patterns in different media. Direct cultures and direct plating techniques can be achieved by plating infected tissue or soil or water filtrates on selective media (Tsao, 1983). The direct plating method has many advantages, such as using pure culture in various studies. However, diagnosis through morphological structure needs skills and a lot of experience. Before the advent of quantitative PCR, the serial dilution method was the easiest way to quantify pathogens from soil or plant tissue (Tsao, 1983; Wakeham & Pettitt, 2017). Many studies have quantified soilborne *Phytophthora* and *Pythium* using the serial dilution plating technique (Kernaghan et al, 2008; Martin & Hancock, 1986; Tsao, 1983).

1.5.2 Molecular approaches to disease diagnosis

1.5.2.1 Immunoassays

Enzyme-linked immunosorbent assay (ELISA) is a simple serological-based diagnostics assay. Engvall and Perlmann first developed ELISA in 1971 by modifying the radioimmunoassay (Aydin, 2015). The researchers used tagged enzyme-based antigen and antibody radioisotopes in radioimmunoassay instead of radioactive iodine 125 to determine the level of immunoglobulin G in rabbit serum (Aydin, 2015). ELISA has very high

specificity because the antibody of any pathogen species binds its antigen or vice versa. A few years later, Clark & Adams (1977) developed ELISA to quantify the plant viruses in the host tissue. Since then, many ELISA assays have been designed to detect potato pathogens, including bacteria, viruses, and oomycetes (Harrison et al, 1990). Different types of ELISA, such as double antibody sandwich ELISA (DAS-ELISA) and plate trapped antigen (PTE ELISA), are used widely in many diagnostic labs. Bacteria and Viruses have been diagnosed mostly by using double antibody sandwich ELISA, whereas fungal and oomycete have been diagnosed using PTE ELISA (Dewey & Thorton, 1995; Wakeham et al, 2004).

Lateral flow immunoassays (LFIA), also called immunochromatographic assays, are an on-site portable diagnostic assay based on species-specific polyclonal antibodies with or without gold particles (Zhan et al, 2018). The history of immunoassay dates to thousand years ago in China, where saliva-based diagnostics were very common during that period (O'Farrell, 2009). However, the technical basis of later flow immunoassay was developed by Plotz and Singer in 1956 from the latex agglutination assay (Wong & Tse, 2008). With the continuous development of enzyme-based immunoassays since 1960, the modern-day antibody/antigen-based lateral flow immunoassays were developed in the late 1980s and commercialized by 2006 (Wong & Tse, 2008). Besides LFIA, another portable immunoassay is immunostrip by Agdia for the preliminary detection of *Phytophthora* genera (Torres et al, 2010). The immunostrip is provided with SEB1 extraction buffer, a special bag, and immunostrip. Samples can be prepared in a minute, and results are obtained in another 1 minute. This immunostrip method is quick, reliable, and affordable for differentiating pathogens at the genus level (Torres et al, 2010). The dot-blot immune-binding assay is another simple diagnostic technique for routine screening of *Phytophthora* and *Pythium* (Hahn, 1997). This technique is the same as other assays; however, the samples are loaded on filter paper or nitrocellulose membrane after mixing the buffer and take up to a half-day to get results (Hahn, 1997).

Isozymes are the multiple forms of an enzyme resulting either from multiple gene loci or multiple alleles at a single gene or secondary modifications (Giles & Ruddle, 1973). The standard and popular method to separate the different isozymes is starch gel, acrylamide gel, agarose gel, cellulose acetate plates on different ionic compositions and pH (Giles & Ruddle, 1973; Nance et al, 1968). The isozyme variation between and within species of potato

pathogens, especially *P. infestans,* was exploited to distinguish variation between them. Isozyme analysis gained popularity until PCR-based techniques (Tooley et al, 1985). Goodwin et al (1995) developed a new cellulose acetate electrophoresis method using the same basic principles to genotype different clonal lineages of *P. infestans* using glucose-6-phosphate isomerase analysis. This isozyme-based genotyping is helpful to monitor the variability among *P. infestans* clones; however, it can give limited information only up to 10 alleles (combined Gpi and Pep). Isozyme analysis is straightforward; the result can be obtained in an hour if the samples are of better quality. The worldwide collection *Pythium ultimum* was genotyped using isozyme analysis in conjunction with morphological characteristics (Barr et al, 1996).

1.5.2.2 Molecular nucleotide assays

The DNA amplification was initially developed by Gobind H. Khorana in early 1960s. Khorana used the synthetic oligonucleotides as primers for DNA polymerase (Khorana et al, 1966). Later in 1983, a biochemist from Cetus Corporation, Kary B. Mullis developed the basic PCR method (Erlich, 1989). Two years later, Cetus Corporation and Perkin-Elmer Biotech Company developed a thermal cycler and PCR reagents used for DNA amplification (Singh et al, 2014). The modern-day PCR technique became more stable and efficient after the discovery of thermostable DNA polymerase from thermophilic bacterium *Thermus aquaticus* (Brock & Freeze, 1969). Thomas D, Brock isolated the thermophilic bacterium and later thermostable enzyme *Taq* DNA polymerase was isolated and used in PCR (Brock & Freeze, 1969; Bryan, 2008; Singh et al, 2014). Since then, many scientists have developed advanced diagnostic assays to detect and diagnose pathogens based on nucleic acid amplification. Modern nucleic acid approaches such as Polymerase Chain Reaction (PCR), real-time PCR, quantitative real-time PCR, Loop-mediated isothermal amplification (LAMP) are the most common methods to detect or identify plant pathogens, including *P. infestans*, *P. erythroseptica* and *P. ultimum* (Böhm et al, 1999; Cullen et al, 2007; Harrison et al, 1990; Judelson et al, 2000; Lees et al, 2012; Ristaino et al, 2001; Trout et al, 1997).

The loop-mediated isothermal amplification assay (LAMP) was initially developed by Notomi et al (2000). LAMP assays use 2 to 3 primer pairs, significantly increasing the amplification of the target DNA and in a shorter period than can be achieved by qPCR. Many

LAMP-based diagnostics tools have been developed to detect *P. infestans* and *Pythium ultimum* on different platforms (Hansen et al, 2016; Khan et al, 2017; Lees et al, 2012, 2019; Ristaino et al, 2019; Shen et al, 2017). These LAMP-based diagnostic assays have been developed to detect pathogens from different sources rapidly and accurately. Many LAMP-based diagnostics assays are designed to run in a heat block or water bath, and results are interpreted based on the change in color of the reaction (Si Ammour et al, 2017; Goto et al, 2009; Hansen et al, 2016; Khan et al, 2017; Ristaino et al, 2019). Optigene Ltd (Horsham, UK) has developed portable, battery-powered instruments to run LAMP reactions, making LAMP even preferable for many diagnosticians.

PCR or real-time PCR is the standard technique that uses a pair of primers, buffer reagents, and thermostable DNA polymerase or *Taq* polymerase to detect, identify or quantify the target pathogens in the samples (Erlich, 1989; Saiki et al, 1988). Many real-time PCR assays have been developed to detect *P. infestans*, *P. erythroseptica*, and *P. ultimum* from different sources with high sensitivity and specificity (Atallah et al, 2006; Böhm et al, 1999; Cullen et al, 2007; Lees et al, 2012; Llorente et al, 2010). PCR and real-time PCR are useful for samples with a low copy of target DNA that immunoassays such as LFDs and immunostrips may not detect. PCR and real-time PCR also have limitations, such as false-positive results because of primer dimers when the primers are not appropriately designed. The target samples might contain contaminants such as humic acid, especially in soil samples which inhibits the reaction and gives a false negative result. With the increasing use of multiplexing to cut down the cost, time, and labor, it is possible to multiplex these three oomycetes using probes labeled with different fluorescent dyes. Multiplexing has a lot of advantages; however, the low concentration or density samples are easily suppressed.

Genotype identification based on morphological characters can be very difficult or almost impossible. Therefore, molecular approaches to distinguish *P. infestans* at clonal lineages or genotype levels were started in the 1990s. DNA fingerprinting probe RG57 is a powerful DNA-based molecular tool that hybridizes to more than 25 different genetic loci of *P. infestans* (Goodwin et al, 1992). Probe RG57 had been used for many years to study the variation within the *P. infestans* population. Later, restriction fragment length polymorphism (RFLP) based markers were developed using different mitochondrial loci to identify different

mitochondrial haplotypes in the *P. infestans* population; thus, genotypes were differentiated (Griffith & Shaw, 1998; Martin et al, 2012).

Phytophthora infestans is heterothallic with two distinct mating types, producing oospore through sexual reproduction. Sexual reproduction has a huge impact on genotype variation and disease development. Interest was shown in a molecular approach besides a pairing test to determine the mating type. Various methods have been developed based on RFLP and amplified fragment length polymorphism (AFLP) to distinguish the mating types of *P. infestans* (Judelson et al, 1996; Kim & Lee, 2002)

Simple sequence repeats (SSR) have been exploited to get in-depth information on the diversity of *P. infestans* (Knapova & Gisi, 2002; Lees et al, 2006; Li et al, 2010; Li et al, 2013). A one-step multiplex PCR method developed with 12 microsatellite markers is helpful to characterize the isolates of *P. infestans* (Li et al, 2013). One-step multiplex is suitable for high-throughput screening of *P. infestans* population as it saves a lot of time, energy, and money. A qPCR-based allele-specific oligonucleotide assay was developed by Gagnon et al (2016) in 2016 using 14 single-nucleotide polymorphisms to identify the major five dominating genotypes of *P. infestans* in Canada. Recently, Hansen et al (2016) has developed a new assay based on genotyping by sequencing (GBS) to characterize the sub-lineages of *P. infestans*. However, this method has already been used in other pathogens (Grünwald, McDonald & Milgroom, 2016). Another molecular approach to detect and identify oomycete pathogens is using a macro array. Membrane-based macro arrays were developed that use at least two specific oligonucleotides per pathogen to detect and identify the oomycete pathogen (Zang, McCarthy & Smart, 2008).

Sequencing of internal transcribed spacer (ITS) is one of the most sequenced DNA of fungus. Generally, ITS 4 and ITS 5 are sequenced to diagnose the oomycetes, including *P. infestans* (Trout et al, 1997). However, other ITS regions can also be used to diagnose oomycetes. Bailey et al (2002) used a unique sequence of ITS1 ribosomal DNA to identify the isolates of *P. infestans* and *P. ultimum* at the species level. Besides the ITS region, the cytochrome oxidase (*cox*) 1 and 2 region of the pathogen is another region of DNA that researchers usually sequence (Grünwald et al, 2011). The recent advanced diagnostics method is next-generation sequencing which provides much information about the sample by

analyzing the combination of short tandem repeats (STR), single nucleotide polymorphism (SNP), and insertion/deletion mRNA (Borsting & Morling, 2015). The whole genome has been sequenced of *P. infestans* (~240 megabases) to study the evolution of this pathogen (Haas et al, 2009). Genotyping of *Phytophthora infestans* can be achieved using sequencing. Recently, *P. infestans* population diversity was investigated using a novel genotyping method by sequencing (Hansen et al, 2016).

1.6 Objectives

Oomycete pathogens pose a big threat to potato production in the USA. Genotypes of *P. infestans* are continually evolving. This causes problems for conventional breeding of resistant cultivars as new strains may evolve which can overcome resistance. Therefore, it is important to study genotypes and population diversity of *P. infestans*.

Phytophthora infestans is destructive pathogen that can destroy the entire potato field within a few days under conducive environment conditions if control measures are not applied on time. Timely diagnosis of *P. infestans* helps growers to use proper disease management strategy, so that they can minimize the loss from late blight. Several diagnostic assays including loop-mediated isothermal amplification assays have been developed to detect *P. infestans*, however, some lack specificity or are less sensitive. Therefore, a rapid, sensitive, and species-specific diagnostic assay needs to be developed to detect *P. infestans* in the field.

Pythium leak and pink rot are the important storage rot diseases causing huge economical loss every year. There are many factors involved from growing stage to harvest and storage of potato that are crucial for the development of the pink rot and Pythium leak in the storage. The factors such as inoculum density in the soil, pre-storage temperature (at harvest) and storage temperatures need to be studied. Since there are very limited chemical control measures of pink rot and Pythium leak in storage, the commercially available in-season foliar fungicides need to be evaluated to control them in storage.

The purpose of this study is to develop an understanding of the genotype and diversity of the *Phytophthora infestans* populations in Bangladesh and Indonesia, an on-site diagnostic assay of *P. infestans*, the effect of inoculum density and pre-storage temperature on Pythium

disease development and in-season foliar fungicides to control Pythium Leak and pink rot in storage.

The main objectives were as follows:

a. to characterize the *Phytophthora infestans* population
b. to develop species-specific on-site diagnostic loop-mediated isothermal amplification assay of *Phytophthora infestans*
c. to determine the effect of soil inoculum level and pre-storage temperature on Pythium leak disease development in storage
d. to evaluate the commercial fungicides applied in season to control Pythium leak and pink rot in storage.

2 MATERIAL AND METHODS

2.1 Introduction

Some same materials and methods have been used throughout this thesis. This chapter has been compiled with common materials and methods used throughout the thesis to reduce redundancy. Materials and methods specific to certain chapters will be discussed in those chapters.

2.2 Growth media

2.2.1 Selective pea media

For 1 L of selective pea media, 160 g of unsalted green garden peas was ground finely in an Oster blender (Oster, USA). The ground pea was filtered using double layer of cheese cloth and 15 g of Bacto agar (VWR International, PA, USA), 5 g of sucrose, and 0.05 g of β-sitosterol was added. The final volume was brought to 1 L distilled water and autoclaved at 15 psi for 30 minutes. The media was amended with 75 mg L^{-1} rifamycin (R), 100 mg L^{-1} ampicillin (A), and 75 mg L^{-1}nystatin (N) dissolved in 1 mL of 100% dimethyl sulfoxide. The antibiotics were added when the media temperature is about 50-60 and poured in the plate. The antibiotic mixture is known as RAN (Forbes, 1997).

2.2.2 Rye A and B media

For 1 L of Rye A agar, 100 g of rye seeds were soaked for 24 hours. The supernatant was saved in beaker for later use. The germinated seeds were placed in a stainless-steel container and 2 L of distilled water was added and boiled for 1 to 1.5 hours. The level of water was checked frequently. The boiled liquid was filtered with 2 layer of cheese cloth. The supernatant was added in the filtered liquid. For 1L 15 g of Bacto agar (VWR International, PA, USA) 10 g of sucrose were added to the liquid/supernatant and autoclaved at 15 psi for 30 minutes. The media was amended with RAN before pouring into the plate.

For Rye B, 15 g of Bacto agar (VWR International, PA, USA), 10 g of sucrose, and 0.05 g of β-sitosterol were added to the liquid/supernatant and autoclaved at 15 psi for 30

minutes. The media was amended with RAN before pouring into the plate

2.2.3 Cornmeal agar

For 1 L of cornmeal agar, 17 g of cornmeal agar powder (VWR International, PA, USA) was added to 1 L of distilled water and autoclaved at 15 psi for 30 minutes. If contamination was an issue, after cooling, the media was amended with 5 mg pimaricin (P), 250 mg ampicillin (A), 10 mg rifampicin (R), 100 mg pentachloronitrobenzene (PCNB), and 10 mg benomyl (B) dissolved in 100% DMSO (PARP+B, Oudemans, 1999).

2.2.4 Potato dextrose agar and potato dextrose broth

For 1 L of potato dextrose agar, 39 g of potato dextrose agar powder (VWR International, PA, USA) was added to 1 L of distilled water and autoclaved at 15 psi for 30 minutes. After, letting the media cool down, it was poured in plates. If there was an issue of contamination, 200 mg of Streptomycin was added before pouring the media.

2.2.5 Vegetable-8 (V-8) selective media

The V-8 juice was transferred to four 50 mL tubes and centrifuged for 2 min at 6000 rpm in Eppendorf centrifuge. The clear liquid was transferred to a container and the precipitate was discarded. For 1L, 100 mL purified V-8 juice was mixed with 2 g of CaCO3, and 15 g Bacto agar (VWR International, PA, USA) and distilled water was added to bring the volume to 1L. The media was autoclaved for 30 minutes at 15 psi. After the media was cool, it was amended with RAN antibiotics and poured in the plate.

2.3 Pathogen isolation and culture

2.3.1 *Phytophthora infestans* and *Phytophthora nicotianae*

The pathogen, *P. infestans*, can be directly isolated from infected sporulating lesions, or the lesion can be transferred to a slice of tuber to encourage mycelial growth. The mycelia from infected tubers can easily be transferred to pea media amended with antibiotics (to avoid bacterial contamination). When mycelia were observed on the tuber surface, a small piece of mycelia was transferred to selective pea media with the help of a sterilized needle or isolation needle. For pure cultures, actively growing hyphal tips or single spores were transferred to pea agar or Rye B agar.

Phytophthora nicotianae was isolated directly from symptomatic potato leaves. Infected leaves were washed several times with distilled water, patted dry with Kim wipes, and air-dried in a Biosafety hood for 10 min (Thermo Scientific). A small piece (~ 1 cm^2) of half infected and half healthy leaf was transferred directly to V-8 selective media. If there were any issues with contamination, the infected leaf tissue was surface sterilized in 5% (w/v) sodium hypochlorite for 1 min and washed several times in autoclaved distilled water. The surface-sterilized leaf tissue was dried as mentioned above. The plates were incubated at room temperature (~21ºC) for 3-5 days. Plates were checked for mycelial growth daily. When mycelia were observed on the plate, a small piece of mycelia or hyphal tip was transferred to V-8 selective media to obtain a pure or sub-culture.

Pure cultures of *P. infestans* and *P. nicotianae* were determined based on morphological characteristics, microscopy, loop-mediated isothermal amplification (LAMP), PCR or qPCR with species-specific primers and finally confirmed by sequencing with ITS4 and ITS5 primer sets. In addition, metalaxyl sensitivity tests were carried out to determine whether isolates of *P. infestans* and *P. nicotianae* were sensitive or resistant to the fungicide metalaxyl using a spiral gradient dilution method (Fairchild *et al*., 2013) and the poison agar method (Forbes, 1997). The genotypes of new isolates of *P. infestans* were further characterized by genotyping using isozyme analysis and simple sequence repeat (SSR) markers. New isolates were labeled with all this information for future use.

2.3.2 *Pythium ultimum* and *Phytophthora erythroseptica*

Pure *Pythium ultimum* and *Phytophthora erythroseptica* cultures were obtained from naturally infected tubers grown in Idaho. A small piece of infected tuber along with healthy tissue was cut and surface sterilized in 5% (w/v) sodium hypochlorite for 1 min. The infected tissue was washed in autoclaved distilled water several times. The infected tissue was air-dried in the biosafety hood for 10 min (Thermo Scientific) and then plated on water agar. The plates were checked the next day for hyphal growth. Hyphal tips were checked under a dissecting microscope and were excised from the media. The hyphal tips were transferred to either cornmeal agar or potato dextrose agar to get pure cultures.

Pure cultures of *P. ultimum* and *P. erythroseptica* were identified based on morphological characteristics and LAMP using species-specific primer sets (for *Pythium*

ultimum). However, sequencing was done to confirm the morphological and LAMP results. ITS4 and ITS5 primer sets were used to sequence these two pathogens. In addition, metalaxyl sensitivity tests were also carried out to determine whether the isolates were sensitive or resistant to metalaxyl using the spiral gradient dilution method (Fairchild *et al*. 2013) and poison agar method (Forbes 1997).

2.3.3 Other *Phytophthora* species

Cultures of some of the *Phytophthora* species used for validating molecular diagnostic assays were not available in our lab. The DNA of these species was obtained from the World Phytophthora Genetic Resource Collection Center (Dr. Coffey, University of California, Riverside). *Phytophthora* species closely related to *P. infestans* and *P. erythroseptica,* such as *P. mirabilis*, *P. andina*, *P. ipomoeae*, and *P. phaseoli,* were used to confirm the specificity of the molecular diagnostics assays for *P. infestans*, *P. erythroseptica,* and *Pythium ultimum*. Detailed information on *Pythium* isolates and all the *Phytophthora* species and *P. infestans* genotypes used in different experiments are listed in Table 2.1.

Table 2.1 Details of species of *Phytophthora*, *Pythium*, and genotypes of *Phytophthora infestans* used in this study.

Isolate/genotype	Species[a]	Source[b]	Host	Origin
US-8	*Phytophthora infestans*	Kirk lab/Simplot†	Potato	Michigan, USA
US-22	*P. infestans*	Kirk lab/Simplot†	Potato	Michigan, USA
US-23	*P. infestans*	Wharton lab/Simplot†	Potato	Idaho, USA
US-24	*P. infestans*	Gevens lab/Simplot†	Potato	Wisconsin, USA
EU 13_A2	*P. infestans*	Cooke lab	Potato	UK
EU 6_A1	*P. infestans*	Cooke lab	Potato	UK
P7722	*P. mirabilis*†	Coffey lab	Tomato	California, USA
P13803	*P. andina*†	Coffey lab	Tamarillo	Ecuador
P10226	*P ipomoeae*†	Coffey lab	unknown	Mexico
P10145	*P. phaseoli*†	Coffey lab	Lima bean	Delaware, USA
Nico-N003	*P. nicotianae*	Mid-Michigan Agronomy, MI	Potato	Indiana, USA
P. erythroseptica	*P. erythroseptica*	Wharton lab	Potato	Idaho, USA
P. ultimum	*Pythium ultimum*	Wharton lab	Potato	Idaho, USA

[a] Species followed by a † were represented by DNA samples only.

Kirk lab = Dr. William Kirk, Michigan State University, East Lansing MI USA.

Wharton lab = Dr. Phillip Wharton, University of Idaho, Aberdeen ID USA.

Gevens lab = Dr. Amanda Gevens, University of Wisconsin Madison WI USA.

Cooke lab = Dr. David Cooke, The James Hutton Institute UK.

Coffey lab = Dr. Michael Coffey, University of California Riverside, CA USA.

Mid-Michigan Agronomy = Robert Schafer, Mid-Michigan Agronomy Inc., MI USA.

2.4 Inoculum preparation

Inoculum of *P. erythroseptica* and *P. ultimum* was prepared either on PDA plates or potato dextrose broth (PDB, VWR International, PA, USA). An actively growing 5 mm mycelial plug was transferred to PDA plates amended with 20 g of CaCO3 to encourage sporulation. The plates were incubated at 20ºC for 14 days. After 14 days, PDA plates were flooded with 10-15 mL of sterilized water. Mycelia and spores (Sporangia or oospores) were dislodged with a glass rod or scraped with a scalpel. The suspension was collected in a 500 mL beaker, macerated in an Oster blender (Oster, USA), and then kept at 4ºC for 4 hours. Twenty actively growing 5 mm mycelial plugs were transferred to a 3 L conical flask (Pyrex) containing 2 L of PDB (20 g per L) to culture the inoculum. The flasks were swirled gently to settle the plugs at the bottom of the flask. The flasks were incubated at 20ºC for 14 days. After 14 days, the cultures in PDB were macerated in an Oster blender and kept at 4ºC for 4 hours before use.

2.4.1 Inoculation procedures

2.4.1.1 Tuber inoculation

In our experiments, potato fields were inoculated with the pathogen suspensions in the field or artificially inoculated in the lab. Infected tubers from inoculated fields were considered naturally infected. Fields were inoculated by spraying PDB inoculum suspensions on plot rows twice in the growing season using a boom sprayer. Fields were inoculated once at the beginning of the season before plant emergence and later about three weeks before vine kill.

In the lab, tubers were bruised by dropping in a three-meter high corrugated metal tower. Tubers were passed through the tower twice. Wounded tubers were then inoculated by immersing in an inoculum suspension of *P. ultimum* or *P. erythroseptica* for 16 h as follows. Thirty-gallon plastic drums were filled with warm water at 23-25 ºC, and inoculum was added to this to create an inoculum suspension. Tubers to be inoculated were placed in onion sacks, and the sacks were immersed in the drums for 16 h. After 16 h, the onion sacks were removed from the inoculum suspension and placed in plastic crates lined with moist burlap bags. Tubers were immersed in sterile distilled water for 16 h for the control treatment. Tubers were incubated either at different temperatures based on the objectives of the experiments. The

moisture of burlap bags was checked every other day, and water was sprayed. The burlap bags were soaked in water, and the crates were covered to maintain humidity.

2.4.2 DNA extraction and purification

2.4.2.1 DNA extraction from pure cultures

Genomic DNA was extracted and purified using a modified cetyltrimethylammonium bromide (CTAB) buffer and Wizard magnetic bead DNA purification kit (Promega, Southampton, UK) using a Kingfisher mL robot (Thermo Scientific, UK). A single leaf infected with *P. infestans* cut into small pieces or approximately 400 mg of mycelia from a pure culture was added to a 5 mL plastic tube with a screw cap containing 2 mL of CTAB tissue extraction buffer and a steel ball bearing. The cap was placed on the tube, and the tissue or mycelia was macerated by shaking the tube and ball bearing. One mL of the suspension was then transferred to a 2 mL centrifuge tube, and 250 µL of lysis buffer B was added from the Wizard kit. The suspension was gently vortexed to mix it properly. After mixing, 750 µL of precipitation buffer was added, and the tube was vortexed briefly to make sure everything was mixed thoroughly. The samples were then centrifuged for 10 min at 14,000 rounds per minute (rpm). Samples were prepared for purification in the Kingfisher mL according to the Wizard magnetic DNA purification system protocol. After extraction and purification with the Kingfisher mL robot, the DNA concentration of the purified samples was determined using a NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). If necessary, the samples were diluted to the desired concentration and stored in a freezer at -20°C until needed.

2.4.2.2 DNA extraction from stems and tubers

A total of five peels from each tuber and any infected tuber tissue was used to extract DNA. Each sample consisted of five tubers. A one-inch portion of the stem from the ground level was cut for potato stem samples. Each sample consisted of 5 stems. The sample was weighed, and tissue was macerated using a juicer (Omega Juicer, FL USA). The juice was collected in Bioreba bags (Bioreba Ag, Switzerland) and used for DNA extraction. The juicer was cleaned with 10% (w/v) sodium hypochlorite (Clorox) and ethanol between sample extractions. Ten mL of CTAB buffer was mixed with stem juice in Bioreba bags (Bioreba Ag, Switzerland). DNA was extracted from stem and skin samples and purified as described

above in section 2.4.2.1 The skin of potato tubers from different trials was peeled using a potato peeler. DNA was extracted using the same method and protocol for all the experiments, including tubers incubated at different pre-storage and storage temperatures and challenge inoculation studies.

2.4.2.3 DNA extraction from soil

The protocol developed by Woodhall et al (2012) was used to extract DNA from soil. Fifty grams of debris-free soil was added to a 250 mL plastic bottle with six steel ball bearings to extract the DNA. CTAB soil buffer (100 mL) and 3 mL antifoam B (aqueous-silicone emulsion, Sigma-Aldrich, Rockville, MD USA) were added to the bottle (Woodhall et al, 2012). The lids of the bottles were screwed on tightly, and the bottles were shaken gently to mix the contents. Bottles were placed in a tight-fitting mold in a 5-gallon paint bucket. The bucket was then placed in a commercial paint shaker and shaken vigorously for 2 min. After grinding the soil to a fine powder in the paint shaker, 40 mL of the clear extract in each bottle was poured (avoiding the foam and debris) into a new 50 mL tube, and the tubes were centrifuged for 3 min at 5000 g. Then 10 mL of the clear extract was transferred to a new tube with 9 mL of 7.5M ammonium acetate. The extract was vortexed, and the tubes were kept on ice for 10 min. The samples were then centrifuged for 3 min at 12000 g. The clear extract was transferred to a new tube containing 2 mL of Lysis buffer B (Promega) and 28 mL of isopropanol. The contents were mixed, and 1.6 ml of acid-washed silica was added. Then the extract was vortexed to mix properly. The samples were centrifuged for 3 min at 12000 g. After centrifugation, the clear supernatant was discarded without dislodging the pellet. The pellet was dried for half an hour at room temperature. The pellet was re-suspended with 1.5 mL TE buffer. The clear extract (750 µL) was transferred to the first well of a Kingfisher rack. DNA was then purified using the procedure described in section 2.4.2.1.

2.5 Quantification of the pathogen on different materials using qPCR

DNA of *P. ultimum* and *P. erythroseptica* from different sources such as stem, tuber, and soil were quantified using species-specific primers (Table 2.6). The primer assays developed by Cullen et al. (2007) were used throughout the study to detect and quantify *P. ultimum* and *P. erythroseptica*. PCR was run in Bio-rad real-time PCR

system (cfx connect real-time system, Bio-rad) or Eppendorf realplex (Eppendorf). The thermocycle conditions were: 10 min at 94°C; 40 cycles of 1 min at 94°C and 60s at 60°C. The final amount of DNA per gram of sample was calculated based on the regression equation from the standard curve, cycle threshold at which the pathogen was detected, and fudge factor.

Table 2.2 Details of the primers and probes sequences used in a quantitative polymerase chain reaction of *Pythium ultimum* and *Phytophthora erythroseptica*.

Pathogen	Primer/Probe	Sequence
Pythium ultimum	Fwd-92	5'-TGTTTTCATTTTTGGACACTGGA -3'
	Rev-166	5'-TCCATCATAACTTGCATTACAACAGA -3'
	Probe-116	5'-CGGGAGTCAGCAGGACGAAGGTTG -3'
Phytophthora erythroseptica	Fwd-99	5' -TGTGCTAGGCTTGGCGTTT -3'
	Rev-177	5' -CCTCGTCCACCCCAGCTTA -3'
	Probe-133	5' -TGCGAAGTAGGGTGTCTGTTCCGGC -3'

Note: These primer assays were developed by Cullen et al, (2007).

3 GENOTYPIC AND PHENOTYPIC CHARATERIZATION OF *PHYTOPHTHORA INFESTANS* POPULATION IN BANGLADESH AND INDONESIA

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3.1 Summary

Late blight caused by *Phytophthora infestans* is an important potato disease that causes significant yield loss in Bangladesh and Indonesia. There were reports of severe outbreaks of late blight for many years in Bangladesh and Indonesia. However, we do not know much about the *P. infestans* populations in Bangladesh and Indonesia. Thus, the objectives of this study were first to identify the genotypes causing late blight in the main potato growing regions of Bangladesh and on the island of Java in Indonesia and second to examine genotypic diversity in the *P. infestans* populations in those regions. A total of 160 samples (n=5 divisions) were collected from both potato (n=140) and tomato (n=20) on FTA cards (n=143) and pure cultures (n=17) in 2018 from Bangladesh and 146 samples were collected on FTA cards (n = 140) or on tubers (n = 6) from 9 regencies of Java Indonesia over 4 years. Late blight samples were characterized based on one-step multiplex microsatellite markers, mating type, mitochondrial haplotype, and metalaxyl-M sensitivity test. We used simple sequence repeat (SSR) data to analyze population and allele diversity statistics, neighbor-joining tree, discriminated analysis of principal components, and minimum spanning network. Microsatellite analysis revealed that EU_13_A2 caused late blight outbreaks in the divisions of Bangladesh with large sub-clonal variations, and the regencies of Java Indonesia were caused by EU_2_A1. We also found other genotypes unique to Indonesia and Bangladesh, with few genotypes close to EU_6_A1 and EU_1_A1. We found a high number

of multilocus genotypes (MLGs) in all nine regencies of Indonesia (131 MLGs out of 146 samples) and Bangladesh (94 MLGs out of 160 samples). The Bangladeshi isolates (n=15) were insensitive to metalaxyl-M, and mating type A2 (n=158) and A1 mating type (n=2), whereas the Indonesian isolates were sensitive or intermediate to metalaxyl-M and mainly A1 mating type. Results indicate ongoing polyploidization in Indonesian *P. infestans* populations due to a high mutation rate and no selection pressure from the susceptible potato hosts grown in Indonesia. These results indicate that in the Bangladesh populations of *P. infestans,* there is large sub-clonal variation, which may be due to different cultivars of potato and hosts being grown in Bangladesh. Since few Indonesian and Bangladeshi isolates were intermediate or insensitive to metalaxyl-M (respectively), alternative disease management strategies need to be adopted.

3.2 Introduction

In Bangladesh, potatoes are grown as a cash crop and used as a staple food. The production per unit area of potato is significantly higher than other major crops grown in Bangladesh, such as rice, wheat, and corn (Azimuddin et al, 2009). These are the main reasons potato production area and yield have increased significantly since 2004 (FAO 2008). Potatoes are grown in Bangladesh by small-scale farmers (~ n=750,000) throughout the plain dry land (Bari) and lowlands after rice production in the winter season. The major potato-growing areas in Bangladesh are northeast and central regions such as Rangpur, Rajshahi, and Mymensingh (Nasif et al, 2018). Diamant, Cardinal, and Granola are Bangladesh's most widely grown potato cultivars (Nasif et al, 2018). Among many diseases and pests present in Bangladesh, late blight is one of the most important and destructive diseases, causing significant yield loss. In the past, yield loss of potatoes in Bangladesh had reached ~ 25-60% depending on cultivars and the onset of disease (Dey et al, 2010; Kessel et al, 2019). Potato growing regions in Bangladesh have foggy and cool winters, which is a conducive environmental condition for late blight.

Indonesia is one of the largest potato producers in Southeast Asia (FAO, 2008). The potato cultivar Granola was bred and released in Germany in the late 1970s and later imported to Indonesia in the early 1980s (Adiyoga, 2009). This cultivar is suitable in Indonesia

because of its medium to high yield potential, early maturity, and adaptation to the Indonesian climate. As a result, Granola is the most widely grown potato cultivar in Indonesia, accounting for 90% of production, and is produced mainly for the fresh market (Adiyoga et al, 1999; Adiyoga, 2009). However, Granola is susceptible to late blight (Adiyoga, 2009). Several factors impact profitable potato production in Indonesia. Among them, late blight is one of the most important due to its impact on crop yield. Yield reductions may be as high as 90%, depending on the crop's growth stage when a disease outbreak occurs (Kusmana, 2003). Potato-growing regions in Indonesia, such as Sumatra and Java, have conducive environmental conditions for late blight year-round, and *P. infestans* are endemic to these regions (Adiyoga, 2009). Infection can occur at any point in the potato crop growth cycle as soon as green plant tissue emerges. Thus, growers in Indonesia rely heavily on fungicides to control late blight, applying as many as 20–30 fungicide sprays in a single potato-growing season (Adiyoga, 2009).

The best way to overcome the problem of late blight in Bangladesh and Indonesia would be to develop a late blight-resistant potato cultivar. Breeding late blight resistance in potato cultivars has significant benefits due to the reduced cost of production through lower fungicide costs, reduced farmer exposure to fungicides, and reduced fungicide residues in food, land, and wastewater (Kirk et al, 2001). Conventional breeding for resistance may take more than ten years to produce a new resistant cultivar. It may no longer be resistant when the new variety is released, as local pathogen strains may have changed. The Feed the Future Biotechnology Potato Partnership was formed in 2015 as a 5-year, $6 million multi-institutional cooperative agreement with USAID to introduce bioengineered potato products in farmer- and consumer-preferred cultivars into Bangladesh and Indonesia. These bioengineered potatoes will have 3 R-gene resistance to late blight. These 3-R genes have been evaluated against *P. infestans* strains from Europe and the USA but not against Indonesian and Bangladeshi isolates. Thus, the objectives of this study were:

i) to identify the genotypes causing late blight in the main potato-growing regions of Java in Indonesia and different divisions of Bangladesh

ii) to examine genotypic diversity in the *P. infestans* populations in the same regions and divisions. Because we have limited information about the *P. infestans* populations in

Bangladesh and Indonesia, this information is critical to ensure the effectiveness and durability of any 3 R-gene cultivars released.

3.3 Materials and methods

3.3.1 Collection of *Phytophthora infestans* samples

DNA samples of *P. infestans* were collected on Whatman Flinders Technology Associates (FTA) cards using the protocol suggested by the manufacturer (Sigma-Aldrich, St Louis, USA). Isolates of *P. infestans* were collected from infected potato or tomato leaves. Potato or tomato leaves with a single sporulating lesion were selected. The infected leaves did not have symptoms of other diseases. Briefly, lesions on infected leaves were placed on top of the cellulose matrix on the FTA card. The paper flap was closed over the leaf using the blunt end of a pen, and the pressure was applied by rubbing to force the juice out of the leaf into the matrix. This deposited the DNA into the specialized matrix. The cards were then allowed to air dry for 30 minutes at room temperature, after which time they were suitable for long-term storage or shipment. FTA cards were stored in a dry place at room temperature until processed for analysis. The FTA cards were shipped to Aberdeen Research and Extension Center, Aberdeen, Idaho.

Some samples were collected on potato slices to isolate pure cultures in the lab. A disease-free potato tuber was cut in half. An infected potato or tomato leaf with a single lesion was placed between the two tuber slices. The two slices of tuber were closed and sealed with parafilm. Tubers with infected leaves sandwiched between the two halves were shipped from Indonesia and Bangladesh to Aberdeen, Idaho. Unfortunately, due to delays in transit, most of the tubers had started to decay by the time they were received, and only a few isolates were obtained from the infected leaves/tubers. The tubers were cut open once the infected leaves/tubers were received at Aberdeen Research and Extension Center, Aberdeen, Idaho lab. The two halves were separated, and any infected part of leaves or tubers were carefully transferred to a new tuber slice. The fresh tuber slice was kept in Pyrex glassware lined with a moist paper towel to maintain humidity. Then the tuber slice in the glassware was incubated at 18 ºC in the dark for 3-5 days. Any mycelial growth on the tuber surface was checked every day and isolated in selective pea media.

A total of 160 samples (from both potato and tomato) were collected from five divisions of Bangladesh (Table 3.1). DNA samples of *P. infestans* (n = 143) were collected on Whatman Flinders Technology Associates (FTA, Sigma-Aldrich, St Louis USA, Table 3.1). Five isolates were isolated on pea agar. Twelve isolates were also directly sent to Aberdeen Research station on pea agar from Bangladesh. A total of 146 samples were collected on FTA cards (n = 140) or tubers (n = 6) from 9 regencies of Java Indonesia over four years. Details about the location and number of samples collected each year from Bangladesh are listed in Fig. 3.1 and Table 3.1, and Indonesia is listed in Fig 3.2 and Table 3.2.

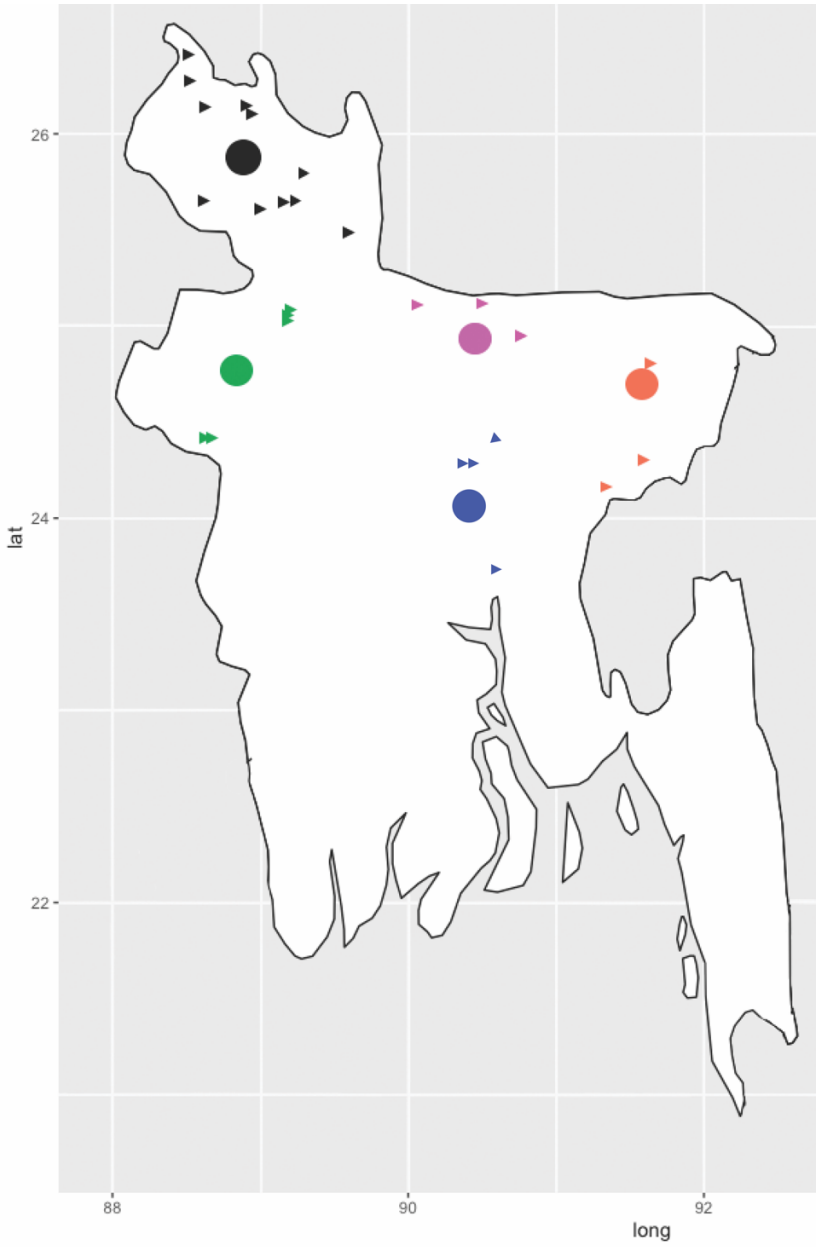


Figure 3.1 Map of Bangladesh showing five divisions out of eight from where samples of *Phytophthora infestans* were collected. (Black solid circle = Rangpur, green solid circle = Rajshahi, purple solid circle = Mymensingh, solid orange circle = Sylhet, and blue solid circle = Dhaka) and different locations (small triangles, the color of triangles corresponds to the division). (Map developed in R).

Figure 3.2 Map of Indonesia (a) and enlarged map of Java (b) showing three different parts (red dots) and nine regencies of Java (blue dots) from where samples of *Phytophthora infestans* were collected. (Map developed in R).

Table 3.1 Geographical information on *Phytophthora infestans* samples (isolates and DNA samples on FTA cards) collected in different years from different divisions of Bangladesh and standard isolates used in the study.

District/ Country	**Locations**[†]	**Total**	**DNA samples or isolates and collection year**
Dhaka	Kishorganj, Joyedebpur, Munshiganj, Gazipur	42	DNA samples = 42, 2018
Mymensingh	Sherpur, Nakla	21	DNA samples = 21, 2018
Rajshahi	Bogora Sadar, Rajshahi	41	DNA samples = 30(Potato), 11(Tomato), 2018
Rangpur	Khaturia, Debiganj, Sadar, Burirhat, Rangpur, Bandarganj, Gaibandha, Panchgarh, Boda, Fultola, Najirerhat	43	DNA samples = 34(Potato), 9(Tomato), 2018
Sylhet	Shibgonj, Madhabpur, North Bejura	13	DNA samples = 13, 2018
US Isolates	Michigan and Idaho	10	Isolates (year = 2008-2015; Genotypes = US-8, US-22, US-23, and US-24)
UK Isolates	NA	2	Isolates from Michigan State University Kirk lab collection (year = 2004; Genotypes = EU_6_A1; EU_13_A2)

*****NA: Information of collection date not available.

[†]All Bangladeshi samples were collected from local subsistence grower's fields (upland and lowland fields). Potato cultivars were Granola, Lady Rosetta, Cardinal, Asterix, BARI-ALU-37, 38, 41, 46, 53, 64, 66, 67, 70, 71, 72, 77, HZD06-1249, Alberta, Diamant, Destiny, Alverston Russet, Ottawa, Surjomukhi, Sayada, Flora, Rosa gold, LB-6, Atlantic, Kalpakri, and tomato cultivars were unknown.

Table 3.2 Geographical information on *Phytophthora infestans* samples (isolates and DNA samples on FTA cards) collected in different years on Java, Indonesia, and standard isolates used in the study.

Country/Region	**Locations**[†]	**Total Samples**	**DNA samples or isolates and collection year**
Bandung	Pangalengan	32	DNA samples = 28, 2016; Isolates = 4, 2019
Batu	Tutungrejo	9	DNA samples = 4, 2018; 5, 2019
Garut	Cisurupan	6	DNA samples, 2016
Majalengka	Argalingga	6	DNA samples, 2016
Mojokerto	Kebonaga	12	DNA samples = 5, 2018; 7, 2019
Pasuruan	Sedaeng, Tosari, Wonokitri, Ngadiwono	25	DNA samples = 11, 2018; 13, 2019; Isolate = 1, 2019
Temanggung	Kledung	10	DNA samples = 8, 2018; 2, 2019
West Bandung	Lembang	10	DNA samples, 2016
Wonosobo	Serang, Parikesit, Tieng, Dieng	36	2018 = 25 DNA samples = 25, 2018; 10, 2019; Isolate = 1, 2019
U.S. isolates	Michigan and Idaho	10	Isolates (year = 2008-2015; Genotypes = US-8, US-22, US-23, and US-24)
EU isolates (UK)	NA	2	Isolates from Michigan State University Kirk lab collection (year = 2004; Genotypes = EU_6_A1; EU_13_A2)

[†] All Indonesian DNA samples and isolates were collected from local subsistence farmers' fields (multiple fields); cultivar is Granola (seed produced in Indonesia); crop rotation is potato-carrot/cabbage.

3.3.2 DNA extraction from FTA cards

FTA cards were processed in two ways, using a QIAmp DNA Investigator kit (Qiagen) or the Whatman FTA purification reagent (GE Healthcare UK). The protocol provided by the manufacturer was used to extract or purify DNA stored on FTA cards. Two 3 mm punches were taken from each FTA card using a Harris cutting pad and a 3 mm puncher (GE Healthcare UK) to purify DNA samples on FTA cards. Punches were stored in a 2 ml microcentrifuge tube. The punches were washed with the FTA purification reagent twice, followed by EDTA or isopropanol, depending on the color of the FTA card. The punches were washed with EDTA if there was no chlorophyll present, otherwise, they were washed with isopropanol. The washed FTA cards were dried for either one hour at room temperature or incubated at 37ºC for 10 min. Washed punches were either stored at -20ºC or 8ºC for no more than seven days before further testing was carried out.

Five 3 mm punches from FTA cards were collected using a Harris cutting pad and a 3 mm puncher. The Qiagen DNA Investigator Kit (Qiagen) protocol was followed with some modifications. Specifically, four punches per sample were used instead of one, and the incubation period was increased to 15 min instead of 10 min. After extraction, DNA was stored at -20ºC for later testing.

3.3.3 Multiplex simple sequence repeat (SSR) genotyping

One-step multiplex simple sequence repeat (SSR) genotyping was performed using 12 microsatellite markers described by Li et al. (2013a; Table 3.3). The Type-it microsatellite PCR kit was used for multiplex amplification (Qiagen, Valencia CA, USA). The protocol by the manufacturer was slightly modified, and PCR was run in 15 µl volume instead of 25 µl. Each PCR reaction contained 7.5 µL of 2x Type-it multiplex PCR master mix, primer concentration described by Li et al (2013a), 1-10 ng template DNA or single purified FTA card, and RNase free water. PCR was run in an Eppendorf thermocycler (Eppendorf, Hauppauge, NY USA). Twelve genotypes of *P. infestans* with known SSR alleles were also included in the PCR. All PCR products from FTA cards were diluted at 1:50 (PCR product: PCR grade water), and genomic DNA was diluted at 1:100.

PCR products were submitted to the Molecular Research Core Facility of Idaho State University (Pocatello, Idaho) for Fragment Analysis. Fragment Analysis was performed in

AB 3130xl Genetic Analyzer (Applied Biosystems). Each sample was processed in line with a size standard Genescan 500 LIZ (Life technologies) for fragment size determination. Optimally diluted samples, 2 µl were combined with 10 µl of HiDi formamide and 500 LIZ size standard (1:20, LIZDye: HiDi) and heat-denatured for three minutes at 95 ºC followed by five min of cooling at 4 ºC. Samples were then processed on the Genetic Analyzer. Raw data and sizing determination were analyzed in GeneMapper software v. 5 (Applied Biosystems, Release 5.0, Build ID FC3, licensed by ISU Molecular Research Core Facility). Allele bins were set for each marker, and alleles were scored.

Table 3.3 List of primer sequences labeled with different dyes used in the multiplex simple sequence genotyping.

SSR Locus	Dye	Product size	Primer sequence
Pig11	NED	130-180	FwdNED-TGCTATTTATCAAGCGTGGG
			Rev-GTTTCAATCTGCAGCCGTAAGA
SSR3	NED	255-275	FwdNED-ACTTGCAGAACTACCGCCC
			Rev-GTTTGACCACTTTCCTCGGTTC
SSR11	NED	325-360	FwdNED-TTAAGCCACGACATGAGCTG
			Rev-GTTTAGACAATTGTTTTGTGGTCGC
D13	FAM	100-185	FwdFAM-TGCCCCCTGCTCACTC
			Rev-GCTCGAATTCATTTTACAGACTTG
SSR8	FAM	250-275	FwdFAM-AATCTGATCGCAACTGAGGG
			Rev-GTTTACAAGATACACACGTCGCTCC
SSR4	FAM	280-305	FwdFAM-TCTTGTTCGAGTATGCGACG
			Rev-GTTTCACTTCGGGAGAAAGGCTTC
Pi04	VIC	160-175	FwdVIC-AGCGGCTTACCGATGG
			Rev-GTTTCAGCGGCTGTTTCGAC
Pi07	VIC	185-205	FwdVIC-ATGAAAATACGTCAATGCTCG
			Rev-CGTTGGATATTTCTATTTCTTCG
SSR6	VIC	230-250	Fwd-GTTTTGGTGGGGCTGAAGTTTT
			RevVIC-TCGCCACAAGATTTATTCCG
Pi63	VIC	265-280	FwdVIC-ATGACGAAGATGAAAGTGAGG
			Rev-CGTATTTTCCTGTTTATCTAACACC
SSR2	PET	165-180	FwdPET-CGACTTCTACATCAACCGGC
			Rev-GTTTGCTTGGACTGCGTCTTTAGC
Pi4B	PET	200-295	FwdPET-AAAATAAAGCCTTTGGTTCA
			Rev-GCAAGCGAGGTTTGTAGATT

The primer sequences listed above were taken from the paper published in the journal of microbiological methods by Li et al, 2013.

3.3.4 Mitochondrial haplotype test

Mitochondrial (mtDNA) haplotypes were determined by PCR- RFLP (Griffith & Shaw, 1998). All isolates were analyzed for mitochondrial haplotypes using the primer pairs P2 F and R and P4 F and R for specific mitochondrial DNA regions P2 and P4, respectively (Table 3.4). Since the mitochondrial haplotype test requires plenty of DNA, DNA samples collected on FTA cards were not or poorly amplified. PCR conditions were 1 cycle of 94°C for 90 s and 40 cycles of 94°C for 40 s, 55°C for 60 s, and 72°C for 90 s, and final extension at 72°C for 15 min. (Griffith & Shaw, 1998). Three to four µl of the amplified PCR product was digested with either *Msp*I (for P2 region) or *Eco*RI (for P4 region) restriction enzymes in a 20 µl volume at 37°C in a heating block for a period lasting between 1 h and overnight (according to manufacturer's instruction). The PCR product was digested with *Msp*I for one to several hours and *Eco*RI for 1 hour (*Eco*RI had greater activity in Promega Multi-Core buffer than in the manufacturer's recommended buffer). The digested PCR products were then mixed with 5 µl of gel-loading buffer, and 15 µl was loaded into a slot on a 2% agarose gel (Gibco BRL Ltd.) in 1 Tris-borate-EDTA (TBE) buffer. The gel was run at 10 V cm^{-1} for 60 to 90 min. Restriction patterns were visualized with a UV transilluminator at 254 nm. Images were recorded using a gel documentation system.

Table 3.4 List of primer sequences used in mitochondrial haplotype determination of *Phytophthora infestans*.

Primer name	Primer sequence
P2F	Fwd 5'-TTCCCTTTGTCCTCTACCGAT-3'
P2R	Rev 5'-TTACGGCGGTTTAGCACATACA-3'
P4F	Fwd 5'-TGGTCATCCAGAGGTTTATGTT-3'
P4R	Rev 5'-CCGATACCGATACCAGCACCAA-3'

3.3.5 Metalaxyl sensitivity test

Isolates of *P. infestans* were tested for metalaxyl sensitivity using the spiral gradient dilution method (Forster et al., 2004; Miles et al., 2014) and the poison agar method (Forbes, 1997). Pea agar media was prepared as described in Chapter 2. In the spiral gradient dilution method, 50 ml pea agar was poured into 100 mm Petri plates. After the medium was set, 10 g/L of metalaxyl-M was prepared from filtered Ridomil Gold EC (479.31 g metalaxyl-M per L; Syngenta Ag). Using an automated spiral plater (Eddy Jet), metalaxyl-M (54 µl) was placed in a spiral gradient on the plate so that the highest concentration of metalaxyl-M (10 g/L) was in the center, with a gradient down to zero concentration at the edge of the plate. After four hours, lines were laid down on the medium from the outside to the inside of the plate with 10 µl of oospores or sporangia and zoosporangia (10^5 sporangia/ml). After seven days, plates were scanned, and the growth of mycelia was measured using Adobe Photoshop and the EC_{50} determined as described by Fairchild et al (2013).

In the poison agar method, aqueous solutions of metalaxyl-M (prepared from Ridomil Gold EC as above) were added to flasks of pea agar (amended with RAN) to give final concentrations of either 0, 5, 10, or 100 mg/L before 25 ml was poured into 50 mm plates. A plug of actively growing *P. infestans* mycelia from a 7-day-old culture was placed in the center of the plate. At 7 and 10 days after plating, radial growth of mycelia was measured, compared with the control culture plate, and sensitivity was calculated as previously described by Forbes (1997).

3.3.6 Mating type test

Three different DNA markers were used to determine the mating type of *P. infestans* isolates from DNA collected on FTA cards, along with a pairing test for living isolates. Isolates were also tested with three DNA markers to see any cross-reaction.

3.3.6.1 Mating type determination based on restriction fragment length plymorphism (RFLP) with W16-1 and W16-2 primers

W16-1 and W16-2 primers for cleaved amplified polymorphic sequence (CAPS) fragment developed by Judelson et al (1995) were used to determine mating types of *P. infestans* samples (Table 3.5). The thermocycle conditions were: 2 min at 94°C; 38 cycles of

45s at 94°C, 60s at 55°C, and 90 s at 72°C, and a final extension for 15 min at 72°C as described by Judelson et al (1995) except the number of cycles were modified to 38 cycles for samples from FTA cards. The PCR products were cleaved with restriction enzyme fast digest *Bsu*R1 using the manufacturer's protocol, and the gel was run using 1.5% agarose gel electrophoresis stained with Gel Red (96V for 20 min, check the gel, and run for more time). Samples with 550 bp and 600 bp were determined to be A1 mating types, whereas samples with only 600 bp were determined to be A2 mating types.

3.3.6.2 Mating type determination based on amplified fragment length polymorphism (AFLP) with Phyb1 and Phyb2

The Second primer set, Phyb1 and Phyb2 primers developed by Kim and Lee (2002), were used to determine the A2 mating types of *P. infestans* samples (Table 3.5). The thermocycle conditions were: 5 min at 94°C followed by 35 cycles of 45s at 94°C, 45 s at 63°C, and 1 min at 72°C, and a final amplification for 15 min at 72°C as described by Kim and Lee (2002). The PCR products were run on a 1% agarose gel and visualized as described above. Phyb1 and Phyb2 marker results in 347 bp product for A2 mating type and no amplification for A1 mating type.

3.3.6.3 Mating type determination based on amplified fragment length polymorphism (AFLP) with S1a and S1b

The Third primer set, S1a, and S1b, developed by Judelson et al (1996), were used to cross-validate the other two DNA markers (Table 3.5). The thermocycle conditions were the same as described by Brylińska et al (2018), except the number of cycles was modified to 38 cycles for samples from FTA cards. The PCR products were run on an agarose gel and visualized as described above. S1b and S2b primers amplified the target fragment and resulted in 1250 bp product for A1 mating type but no amplification of A2 mating type isolates.

Brylińska et al (2018) have reported that isolates were not completely (100%) assigned to specific mating types using these molecular markers (CAPS and Phyb assigning isolates correctly by 96% and 84%, respectively). Hence, she recommended that local populations be validated with the pairing test method before these markers are used for DNA samples.

Table 3.5 List of primer sequences used in mating type determination of *Phytophthora infestans*.

Primer name	Primer sequence	References
W16-1	5'- AACACGCACAAGGCATATAAATGTA -3'	Judelson *et al*. (1995)
W16-2	5'- GCGTAATGTAGCGTAACAGCTCTC -3'	
Phyb-1	5'- GATCGGATTAGTCA GACGAG -3'	Kim and Lee (2002)
Phyb-2	5'-GCGTCTGC AAGGCGCATTTT -3'	
S1a	5'-AGGATTTCAACAA -3'	Judelson *et al*. (1996)
S1b	5'-TGCTTCCTAAGG -3'	

3.3.6.4 Mating type determination based on pairing test

Pairing tests were done for live cultures, as described by Forbes (1997). Unknown isolates were paired with known isolates of either A1 or A2 mating on pea agar. Five-millimeter circular disks of actively growing mycelia (7 days old) from a known and unknown isolate were placed in the center of the plate (pea media), separated by 2-3 cm. The cultures were stored in the dark at 18ºC for 18 days, and the formation of any oospores was checked under a microscope. If the known isolate was A1 and oospores were present, the mating type was determined to be A2, and conversely, if the known isolate was A2 and oospores were formed, then the unknown isolate was determined to be A1.

3.3.7 Data preparation and analysis

All the samples from Bangladesh were amplified by PCR, and hence we got microsatellite data from all 160 samples. Almost all the samples were triploids (n=157) and diploids (n=3; Figure 3.3). The Indonesian samples were ninety percent triploid (n=131), with few diploids (n=11) and tetraploids (n=4; Figure 3.4). Since we had a lot of triploid data, we needed make the data uniform and polyploid, so a zero was added to missing alleles of diploids and triploids. Multiple years data from the same location were combined and analyzed based on geographical location and host. Standard isolates and some already published data. (Table 3.1; Li et al. 2013a; Dey et al. 2018) were compared with Bangladeshi sample data. Indonesian samples, standard isolate (Table 3.2), few Indonesian samples data kindly provided by Dr. David Cooke (The James Hutton Institute, Invergowrie, Dundee, UK), and some previously published data by Li et al. (2013a) were compared for SSR fingerprints to assign specific genotypes. These data were also included in frequency-based analysis samples such as neighbor-joining tree and minimum spanning network to see how our data correlated with them. Data were analyzed in R package *poppr* V.2.3.0., polysat, genodive software described by the authors (Gru¨nwald et al. 2017a, b; Kamvar et al. 2014, 2015, Shakya et al. 2018).

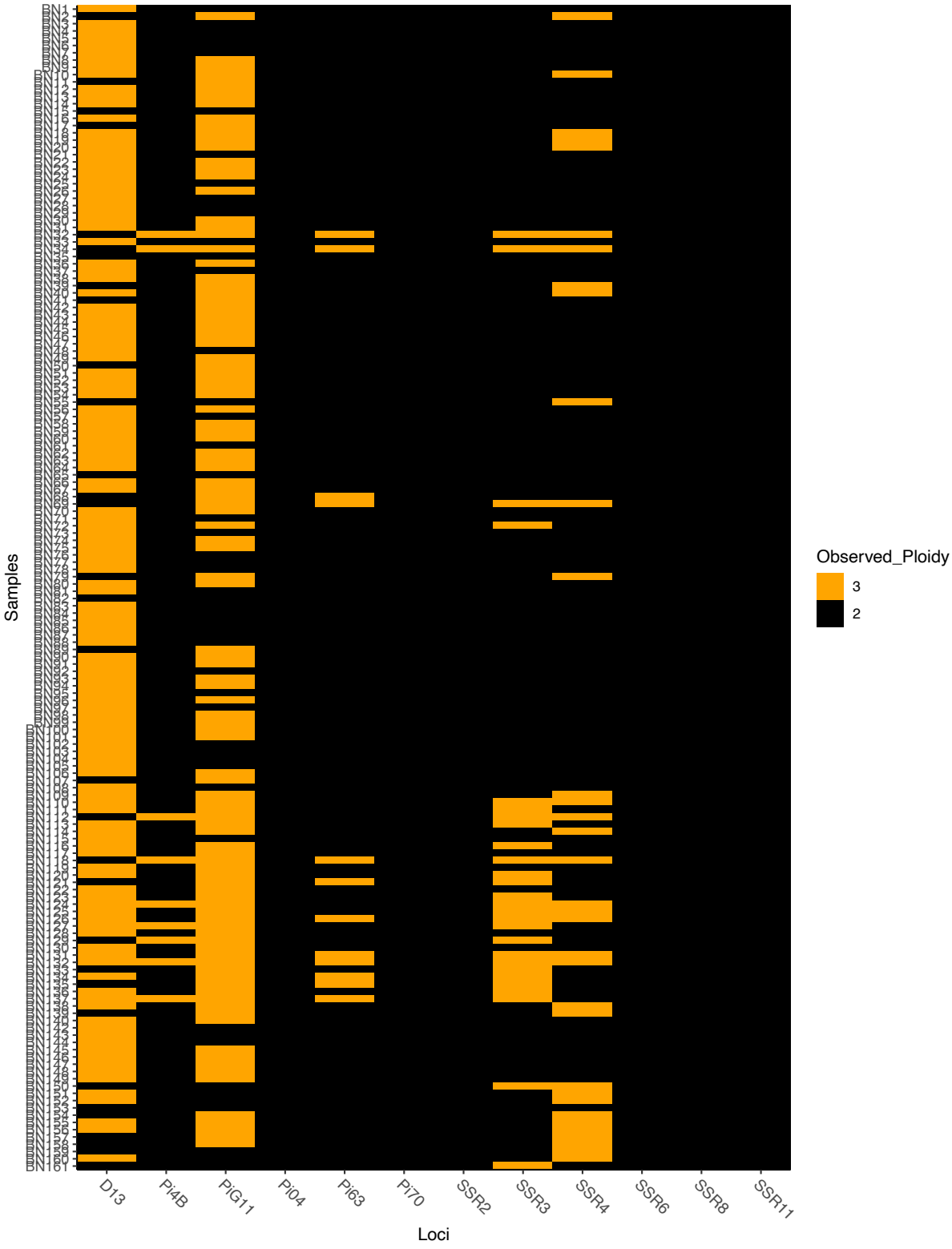


Figure 3.3 Illustration of the ploidy level of clone corrected data of Bangladesh samples at each locus (Yellow are triploid and black are diploid alleles at each locus).

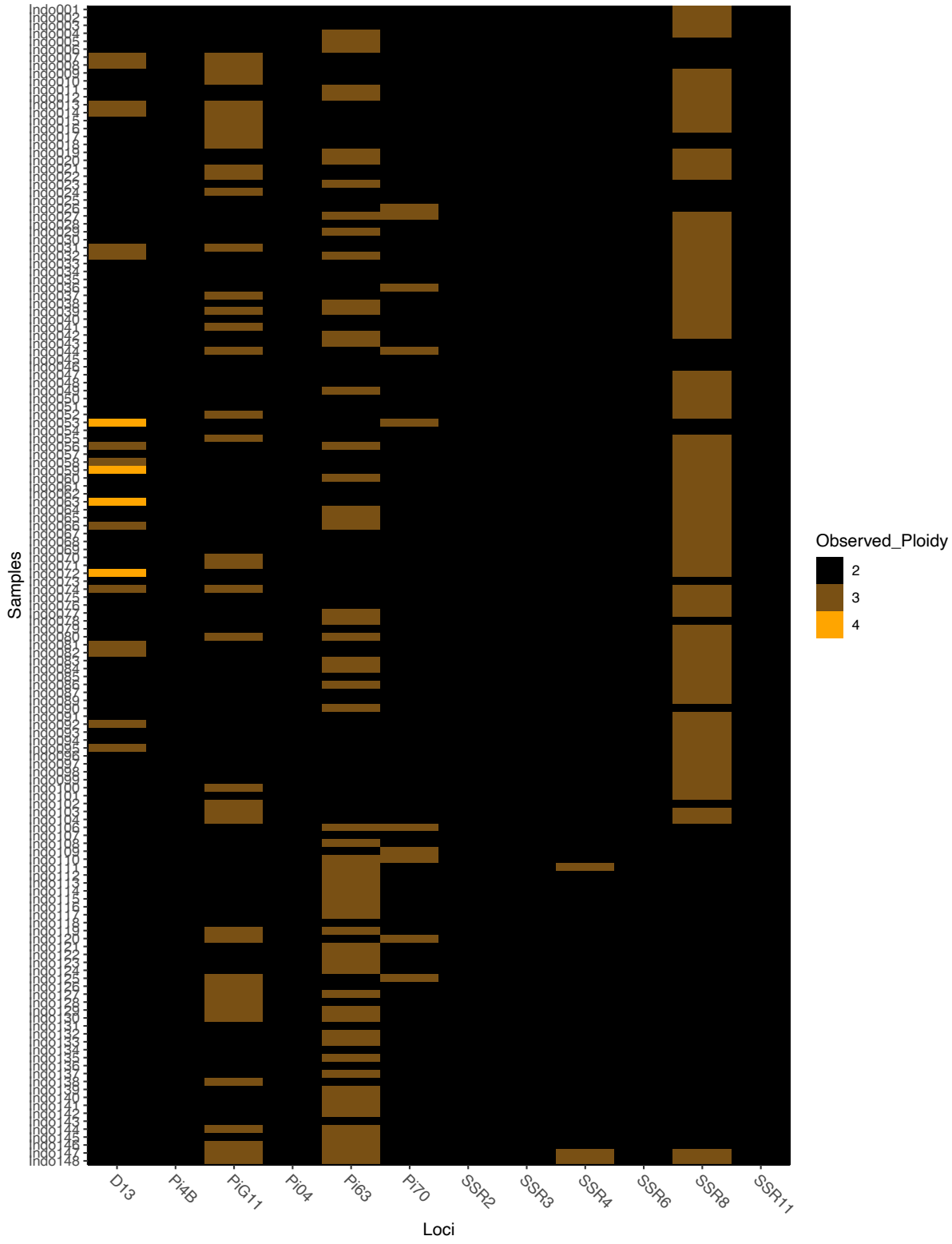


Figure 3.4 Illustration of the ploidy level of clone corrected data of Indonesian samples at each locus (Yellow are tetraploid, dark brown are triploid, and black are diploid alleles at each locus).

3.3.8 Multilocus genotype analysis, population diversity statistics, and mode of reproduction

Multilocus genotype (MLG), estimated multilocus genotypes (eMLG) after rarefaction, and diversity statistics for 160 (Bangladesh) and 146 (Indonesia) polyploid data for 12 microsatellite loci were calculated using the R package *poppr* V.2.3.0 (Kamvar et al., 2014, 2015; R Core Team, 2016; Grünwald et al., 2017). MLG diversity of a population was estimated using Shannon–Weiner Index in R package *poppr* V.2.3.0 (Shannon and Weaver, 1949; Kamvar et al., 2014, 2015). The expected heterozygosity (Hexp) was computed for all samples based on Nei's unbiased gene diversity (Nei, 1978). The mode of reproduction in each population was estimated based on the index of association (IA) and standardized index of association value ($\bar{r}d$; Agapow and Burt, 2001; Kamvar et al., 2014, 2015). We used both clone corrected and non-clone-corrected data to calculate $\bar{r}d$, and the significance was tested with 999 permutations in the R package poppr (Kamvar et al., 2014, 2015). Allelic diversity was calculated based on Simpson's index and Nei's unbiased gene diversity in R package *poppr* V.2.3.0. (Simpson,1949; Nei 1978; Kamvar et al. 2014, 2015).

3.3.9 Population structure and differentiation

The genetic distance between the individual samples was computed using a stepwise mutation model described by Bruvo et al. (2004). Minimum spanning network (msn) and neighbor-joining tree (nj tree) were constructed in *poppr* V.2.3.0., based on Bruvo's distance. A neighbor-joining (nj) tree was constructed with 1000 bootstraps and viewed and modified on Fig tree v1.4.3 (Kamvar et al. 2014, 2015; http://tree.bio.ed.ac.uk/software/figtree/). NJ trees are helpful to visualize how samples cluster and group together compared to the standard samples. Discriminated analysis of principal components (DAPC) was performed in R-package *adegenet* V.2.0.1 to examine how the individual samples cluster to a population (Jombart 2008; Jombart et al. 2010).

Pairwise fixation indices (FST) were calculated using Bruvo's genetic distance using clone-corrected data and R package strataG v. 1.0.5 or polysat (Archer et al., 2017). The FST values were used to examine the differentiation between the populations separated by geographical locations. Analysis of molecular variance (AMOVA) was performed based on Bruvo's genetic distance on clone-corrected data in R package ade4 v. 1.7-5 or genodive

(Excoffier et al., 1992; Dray and Dufour, 2007). AMOVA estimates variance due to individual samples within the population or among populations.

3.4 Results

3.4.1 Mating type, metalaxyl-M sensitivity test, and mitochondrial haplotype of Bangladeshi samples

Most of the samples on FTA cards were positively amplified with CAPS markers and determined to be the A2 mating type (Table 3.6, Figure 3.5). We cross-checked the samples with pairing test (live cultures), Phyb1/Phyb2 and S1b/S2b markers, and the results were A2 mating type except for two samples. Seventeen isolates were also tested for mating type using the pairing test and CAPS markers and determined to be A2 mating type. Out of 17 isolates tested for metalaxyl-M sensitivity, only two isolates were intermediate, and 15 of them were insensitive (Table 3.6, Figure 3.6). See table 3.6 for the definition of categories used in the metalaxyl sensitivity test. All the isolates tested (n=17 from cultures) for mitochondrial haplotype all were Ia haplotype (3.7).

Table 3.6 Phenotypic characterization of *Phytophthora infestans* samples from five districts of Bangladesh.

Samples/Location_host	**No. of DNA samples or isolates**	**Mating Type**	**Metalaxyl-M Sensitivity Test[b]**	**Mitochondrial haplotype**
BN 145-149/Dhaka_P[a]	5	A2 (PT)	Resistant	Ia
BN 16-18, 42-54, 56, 77-88, 116-123/Dhaka_P	37	A2 (PCR)	NA[c]	NA
BN 150, 152-161/Mymensingh_P[a]	11	A2 (PT)	9 = Resistant 2 = Intermediate	Ia
BN 3-7, 20, 21, 89-91/Mymensingh_P	10	A2 (PCR)	NA	NA
BN 38-41, 57, 65-69, 96-115/Rajshahi_P	30	A2 (PCR)	NA	NA
BN 133-140, 142-144/Rajshahi_T	11	A2 (PCR)	NA	NA
BN151/Rangpur_P[a]	1	A2 (PT)	Resistant	Ia
BN 8-13, 19, 22, 26-30,33, 35-37, 55, 58-61, 71-76, 92, 93, 95/Rangpur_P	31	A2 (PCR)	NA	NA
BN 124-132/Rangpur_T	9	A2 (PCR)	NA	NA
BN 32, 34/Rangpur_P	2	A1 (PCR)	NA	NA
BN 1, 2, 14-15, 23-25,31, 62-64,70,94/Syllhet_P	13	A2 (PCR)	NA	NA

[a]Cultures tested for metalaxyl-M sensitivity test and mitochondrial haplotype

[b]Metalaxyl-M Sensitivity: Resistant = growth on both 5 and 100 mg/L poison agar plates with more than 40% growth compared to control (0 mg/L); Intermediate = growth on 5 mg/L plate with more than 40% growth compared to control (0 mg/L); Sensitive = less than 40% growth on both 5 and 100 mg/L plates compared to control (0 mg/L)

PT = Pairing test

PCR = CAPS markers used

[c]NA = Not Available as only the DNA of the samples was collected on FTA cards. (There is no BN141)

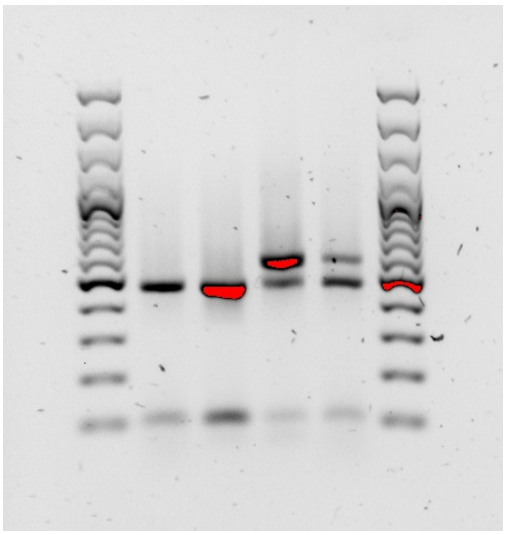
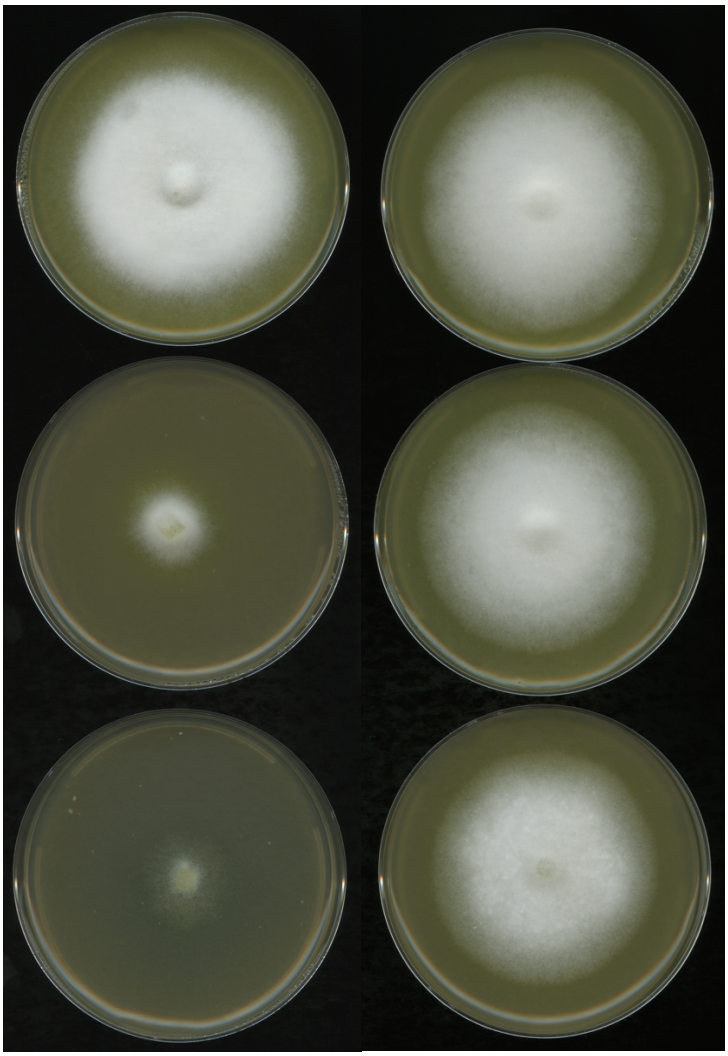
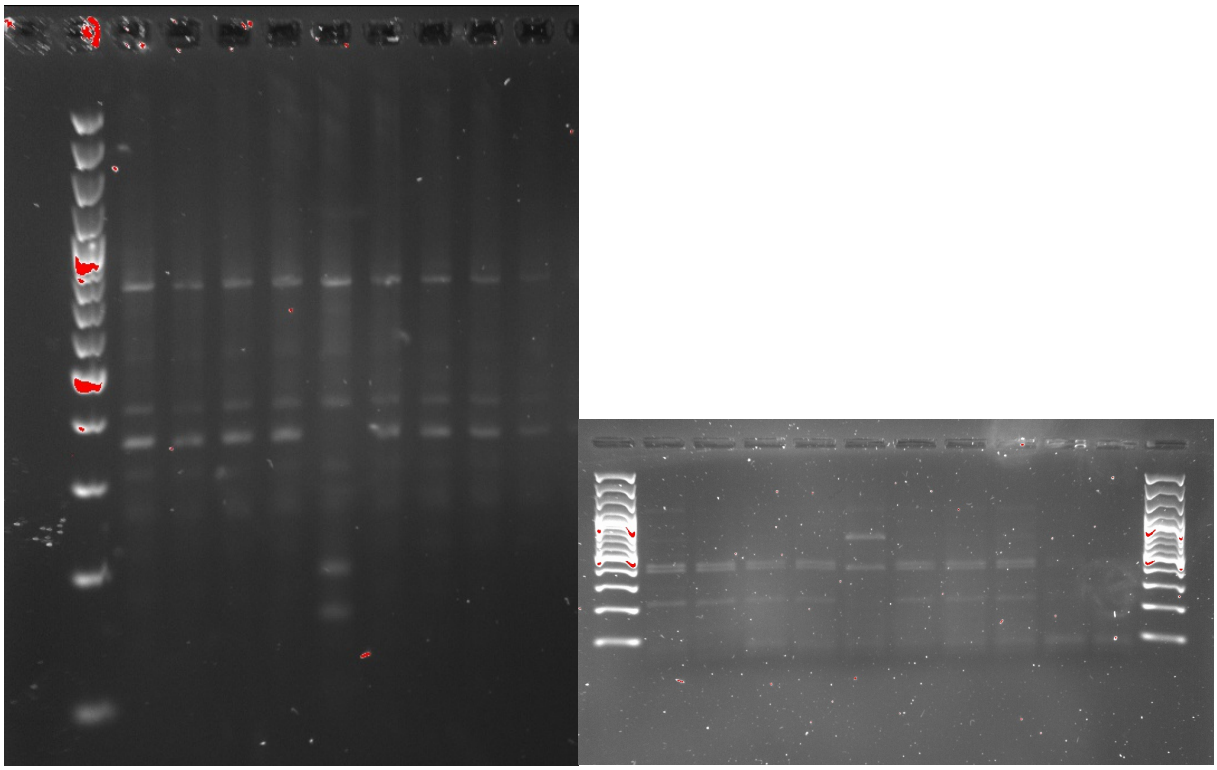


Figure 3.5 Gel of Cleaved Amplified Polymorphism Sequence fragments (from left to right: EU_13_A2, Bangladesh isolate (EU_13_A2), EU_6_A1 and US-23) amplified with W16-1 and W16-2 primer pairs and cleaved with HaeIII restriction enzyme. Marker = 100bp (Each band in ladder/marker is 100 bp)**.** The two bands mean A1 mating type (~ 550 and 600 bp) and one band means A2 mating type (~550 bp).



2a. 2b.

Figure 3.6 Metalaxyl-M sensitivity test of Bangladesh isolate using poison agar method 2a. standard sensitive EU_6_A1 isolate (from top to bottom = plates amended with 0 µg/L, 5 µg/L and 100 µg/L) and 2b. resistant isolates from Mymensingh (from top to bottom = plates amended with 0 µg/L, 5 µg/L, and 100 µg/L). Metalaxyl-M resistant Mymensingh isolate showing full growth on Metalaxyl-M amended media at 5 µg/L and 100 µg/L.



7 a. 7 b.

Figure 3.7 Gel of P2 and P4 products of reference and Bangladesh isolates [from left to right US-8, US-22, US-23, US-24, EU_6_A1, EU_13_A2, Bangladesh isolates (EU_13_A2)] amplified with F2-R2 primer pairs (cleaved with Msp1 enzyme). 3a. and 3b. F4-R4 primer pairs (cleaved with *Eco*R1 enzyme). Marker=100bp.

3.4.2 Mating type and metalaxyl-M sensitivity test of Indonesian samples

Eighty percent of DNA samples were positively amplified with CAPS markers and determined to be the A1 mating type (Table 3.7, Figure). One sample (Indo 111) was identified as an A2 mating type. However, the mating type could only be determined using CAPS and could not be confirmed using the Phyb markers. Six isolates were also tested for mating type using the pairing test and CAPS markers and determined to be A1 mating type. Out of the six isolates tested for metalaxyl- M sensitivity, two isolates, Pasuruan and Wonosobo, were intermediate and four, from Bandung, were sensitive. See Table 3.7 for the definition of categories used in the metalaxyl sensitivity test.

Table 3.7 Phenotypic characterization of *Phytophthora infestans* isolates from nine regencies of Java, Indonesia.

Isolates/Location	**No. of DNA samples or isolates**	**Mating Type**	**Mating Type determination methods**	**Metalaxyl-M Sensitivity[b]**
Indo147/Wonosobo	1	A1	Pairing test/PCR	Intermediate
Indo 148/Pasuruan	1	A1	Pairing test/PCR	Intermediate
Indo 143-146/Bandung	4	A1	Pairing test/PCR	Sensitive
Indo 1 – 28/Bandung	28 (NAmp[a] = 5)	A1	PCR	NA[c]
Indo 29-34/Garut	6	A1	PCR	NA
Indo 35-40/Majalengka	6 (NAmp =1)	A1	PCR	NA
Indo 41-50/West Bandung	10 (NAmp = 1)	A1	PCR	NA
Indo 51-69, 75-80, 131-140/Wonosobo	35 (NAmp =10)	A1	PCR	NA
Indo 70-74, 99-104, 118-130/Pasuruan	24 (NAmp = 5)	A1	PCR	NA
Indo 83-87, 111-117/Mojokerto	12 (NAmp = 1)	A1	PCR	NA
Indo 91-98, 141-142/Temanggung	10 (NAmp = 2)	A1	PCR	NA
Indo 81-82, 89-90, 106-110/Batu	9 (NAmp =2)	A1	PCR	NA

[a]NAmp = Not amplified in PCR and mating type not known

[b]Metalaxyl-M Sensitivity: Resistant = growth on both 5 and 100 mg/L poison agar plates with more than 40% growth compared to control (0 mg/L); Intermediate = growth on 5 mg/L plate with more than 40% growth compared to control (0 mg/L); Sensitive = less than 40% growth on both 5 and 100 mg/L plates compared to control (0 mg/L)

[c]NA = Not Available as only the DNA of the samples was collected on FTA cards.

3.4.3 SSR fingerprints and population diversity statistics of Bangladeshi late blight samples

We compared our SSR data with already published sources and found that almost all samples were similar to worldwide EU_13_A2. Mitochondrial haplotype tested for the 17 isolates revealed our samples and EU_13_A2 had the same Ia haplotype (Figure 7). Based on SSR allele comparison with standard EU_13_A2 from more than 40 isolates published by different authors, haplotype test, and other phenotypic characteristics such as mating type and metalaxyl_M sensitivity test, we designated our samples as EU_13_A2 genotype and its sub-clones. Since most of our samples were clustered with worldwide standard EU_13_A2 in the nj tree, these results also supported our designation of samples as EU_13_A2. Few samples shared the same allele profile as the standard EU_1_A1 and EU_6_A1 and were clustered with these standard isolates in the nj tree, and we designated them as EU_6_A1 or EU_1_A1 (Figure 3.11).

Genotypic diversity of individuals in a population was evaluated, and a total of 94 multilocus genotypes were determined out of 160 samples. The samples in Rajshahi_P (potato) had higher MLGs (26) and were more diverse compared to other divisions, followed by Dhaka (potato, 24) and Rangpur_P (potato, 20). Samples from tomato (Rajshahi and Rangpur, n = 20) were also diverse (MLGs = 17). Isolate diversity was even in many divisions with a value of 1.0 (Table 3.8). All of the divisions had a standardized index of association value greater than zero supporting the evidence of clonal reproduction in all divisions. An index of association with zero indicates sexual reproduction, whereas any value other than zero suggests asexual or clonal reproduction. Rangpur_P, Dhaka_P, and Rajshahi_P had a greater Shannon-Weiner Index value, indicating a high diversity and that these divisions had a higher number of MLGs than the other divisions.

Table 3.8 Multilocus genotype and diversity statistics for microsatellite data for 12 microsatellite loci in populations of *Phytophthora infestans* from Bangladesh by divisions and hosts collected in 2018.

Population (state and host)	**N[a]**	**MLG[b]**	**eMLG[c](SE)**	**H[d]**	**Hexp[e]**	**Evenness**	**Ia[f]**	**rbarD[g]**
Dhaka_P*	42	25	8.11 (1.13)	2.91	0.439	0.681	1.453	0.2161
Mymensingh_P	21	16	8.13 (1.04)	2.53	0.439	0.660	0.406	0.0755
Rajshahi_P	30	26	9.59 (0.58)	3.22	0.462	0.948	0.665	0.0993
Rajshahi_T	11	9	8.27 (0.45)	2.10	0.519	0.855	0.792	0.0818
Rangpur_P	34	20	7.95 (1.12)	2.73	0.465	0.738	3.938	0.4508
Rangpur_T	9	9	9.00 (0.00)	2.20	0.541	1.000	1.136	0.1154
Sylhet_P	13	11	8.85 (0.64)	2.35	0.436	0.941	0.196	0.0685
Total	160	94	9.05 (0.94)	4.09	0.462	0.523	2.047	0.2227
Population (host)								
Potato	140	79	16.30 (1.68)	3.92	0.449	0.530	1.688	0.204
Tomato	20	17	17.00 (0.00)	2.72	0.522	0.812	0.991	0.101
Total	160	94	16.70 (1.64)	4.09	0.462	0.532	2.047	0.223

[a]N: number of individuals per division

[b]MLG: number of multilocus genotypes

[c]eMLG: expected number of MLG for each division (SE: standard error)

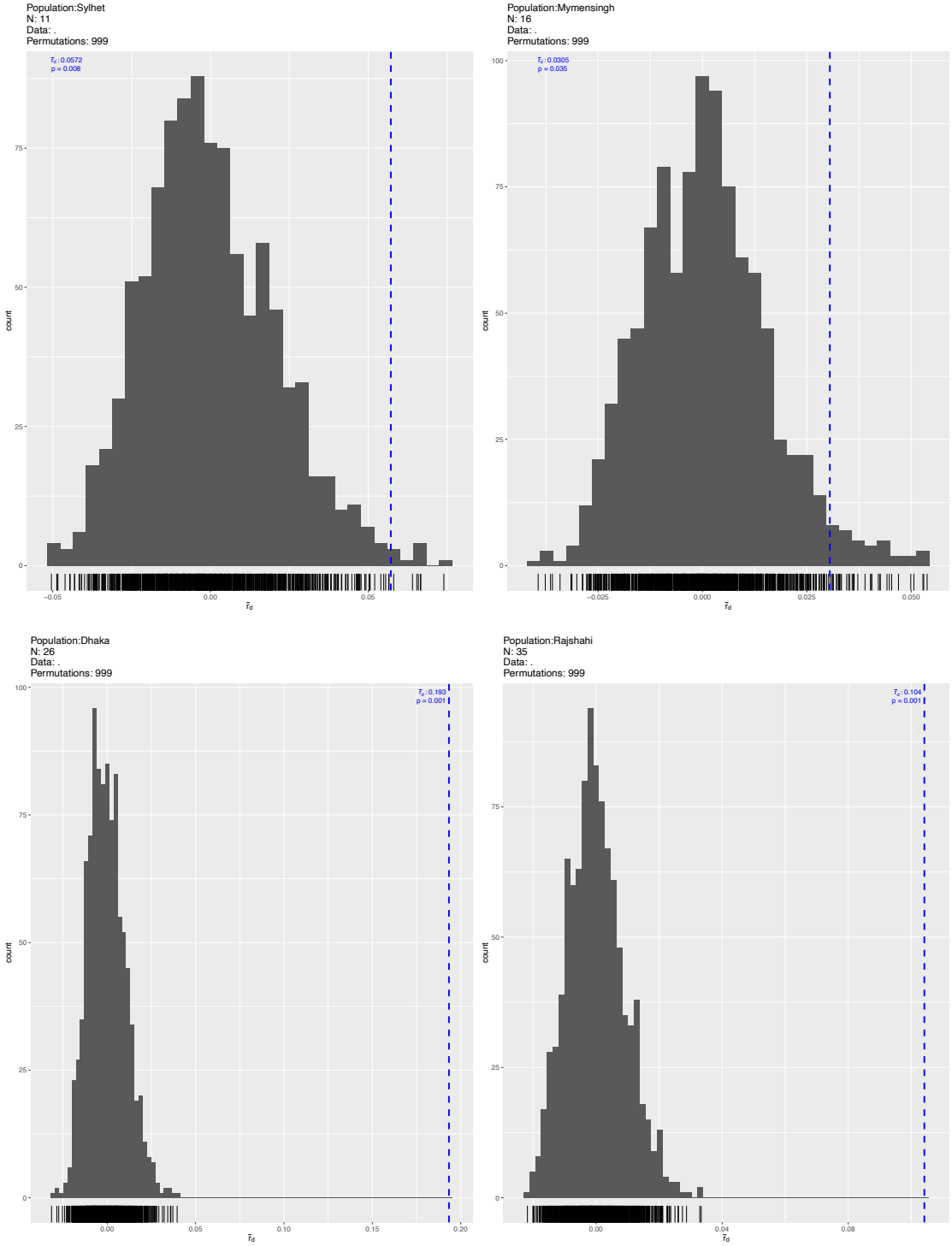
[d]H: Shannon-Weiner Index of MLG diversity

[e]Hexp: Nei's unbiased gene diversity

[f]Ia: Index of association

[g]rbarD: Standardized index of association

*Divisions followed by P = samples collected from potatoes and T = samples collected from tomatoes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p = 0.008
Population:Mymensingh
N: 16
Data: .
Permutations: 999
$\bar{r}_d$: 0.0305
p = 0.035
Population:Dhaka
N: 26
Data: .
Permutations: 999
$\bar{r}_d$: 0.193
p = 0.001
Population:Rajshahi
N: 35
Data: .
Permutations: 999
$\bar{r}_d$: 0.104
p = 0.001
count
$\bar{r}_d$

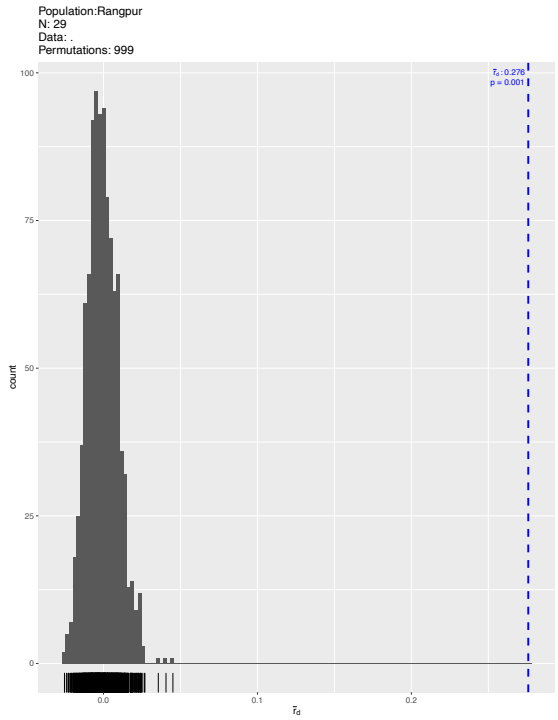


Figure 3.8 Mode of reproduction of *Phytophthora infestans* populations in five divisions of Bangladesh (clone corrected data for 12 microsatellite loci) based on the standardized index of association (rbarD). The p-value of rbarD (blue line) was observed in each population after clone correction. Since *p*-value is < 0.05% in all divisions, we fail to reject null hypothesis which is there is no linkage among locus/markers. No linkage among locus/markers supports the evidence for clonal population.

Allelic diversity of 160 samples based on geographic distribution was calculated using *poppr* V.2.3.0. A total of 49 different alleles were detected from 160 samples ranging from 2 (Pi04, Pi70, and SSR2) to 13 (D13) alleles with a mean of 4.083 alleles per locus (Table 3.9). Gene diversity was estimated using Simpson's index and ranged from 0.043 (SSR2) to 0.798 (D13), indicating that the SSR2 locus was the least diverse and D13 was the most diverse locus. Nei's unbiased estimation was correlated with Simpson's index. Evenness values ranged from 0.403 (SSR2) to 1.00 (Pi04). The D13 locus had the highest number of alleles (13) and high allelic diversity with moderate distribution (0.744, least = 0.403, highest = 1.00), resulting in many MLGs. PiG11(7) also had a higher number of alleles than the rest of the loci except D13, with higher allelic diversity and moderate evenness (0.815) contributing to a larger number of MLGs. Evenness value 1 indicates a single species/genotype in a population (single MLG), and 0 indicates all individuals are unique (highest number of MLGs or number of samples = number of MLGs).

None of the divisions had a *p*-value greater than 0.01. Any *p*-value greater than 0.01 is not significant and fails to reject the null hypothesis (no linkage among markers). None of the Bangladeshi *P. infestans* population had a *p*-value greater than 0.01 for rbarD (0.235), indicating no linkage among markers. Linkage among markers suggests a sexual mode of reproduction. Since there was no linkage among markers this supports the evidence for clonal reproduction in Bangladesh populations.

Table 3.9 Allele diversity for clone corrected microsatellite data for 12 microsatellite loci in populations of *Phytophthora infestans* from Bangladesh.

Locus	**Allele**	**1-D[a]**	**Hexp[b]**	**Evenness**
D13	13.0	0.798	0.800	0.744
Pi4B	3.0	0.532	0.533	0.900
PiG11	7.0	0.694	0.696	0.815
Pi04	2.0	0.500	0.502	1.000
Pi63	3.0	0.532	0.533	0.900
Pi70	2.0	0.123	0.123	0.510
SSR2	2.0	0.043	0.043	0.403
SSR3	4.0	0.567	0.569	0.868
SSR4	4.0	0.637	0.639	0.921
SSR6	3.0	0.498	0.499	0.965
SSR8	3.0	0.527	0.529	0.888
SSR11	3.0	0.072	0.073	0.419
Mean	4.083	0.460	0.461	0.778

[a]1-D: Simpson index, [b]Hexp: Nei's 1978 gene diversity.

3.4.4 SSR fingerprints and population diversity statistics of Indonesian late blight samples

SSR fingerprints of our DNA samples were compared with standard data (our data, data provided by Dr. David Cooke, and data published by Li et al., 2013). The specific genotypes were assigned based on the same or close fingerprints. The specific genotypes were EU_2_A1 (60%), EU_4_A1, and EU_13_A2 (1.5%). The rest of the DNA samples were unique but closer to European genotypes than US genotypes. An NJ tree was constructed, including standard isolates; our assigned genotypes clustered together with EU_2_A1, EU_4_A1, EU_13_A2, or separately (Figure 3.14). Mating type results also supported the assigned genotypes.

The genotypic diversity of individuals in a population was evaluated, and a total of 131 MLGs were determined out of 146 DNA samples. The DNA samples from Wonosobo had a significant number of MLGs (36) and were more diverse compared to other regencies, followed by Pasuruan (24) and Bandung (23). DNA samples from Garut (6) and Majalengka (6) were the least diverse. DNA sample diversity was even in many regencies, with a value of 1.0 (Table 3.10). All regencies had a standardized index of association value greater than zero except Majalengka ($\bar{r}d = -0.0235$, $p < .724$; Figure 3.3), supporting the evidence of clonal reproduction in eight regencies. An index of association with a zero value indicates sexual reproduction, whereas any value other than zero suggests asexual reproduction. Wonosobo, Pasuruan, and Bandung had a greater Shannon–Weiner index value than the other regencies, indicating a high diversity, and these regencies had a higher number of MLGs.

Table 3.10 Multilocus genotype and diversity statistics for microsatellite data for 12 microsatellite loci in populations of *Phytophthora infestans* from Indonesia by Regency collected from 2016 to 2019.

Population	**N[a]**	**MLG[b]**	**eMLG[c] (SE)**	**H[d]**	**Hexp[e]**	**Evenness**	**Ia[f]**	**rbarD[g]**
Bandung	32	23	9.18 (0.75)	3.08	0.597	0.943	0.781	0.0913
Batu	9	9	9.00 (0.00)	2.20	0.589	1.000	1.245	0.1989
Garut	6	6	6.00 (0.00)	1.79	0.587	1.000	1.261	0.1977
Majalengka	6	6	6.00 (0.00)	1.79	0.557	1.000	-0.111	-0.0235
Mojokerto	12	10	8.50 (0.58)	2.21	0.594	0.862	1.249	0.1479
Pasuruan	25	24	9.85 (0.00)	3.16	0.517	0.978	0.436	0.0596
Temanggung	10	10	10.00 (0.00)	2.30	0.565	1.000	1.917	0.2078
West Bandung	10	10	10.00 (0.00)	2.30	0.544	1.000	0.210	0.0451
Wonosobo	36	36	10.00 (0.00)	3.58	0.606	1.000	1.092	0.1162
Total	146	131	9.93 (0.26)	3.97	0.601	0.949	0.656	0.0662

[a]N, number of individuals per regency

[b]MLG, number of multilocus genotypes

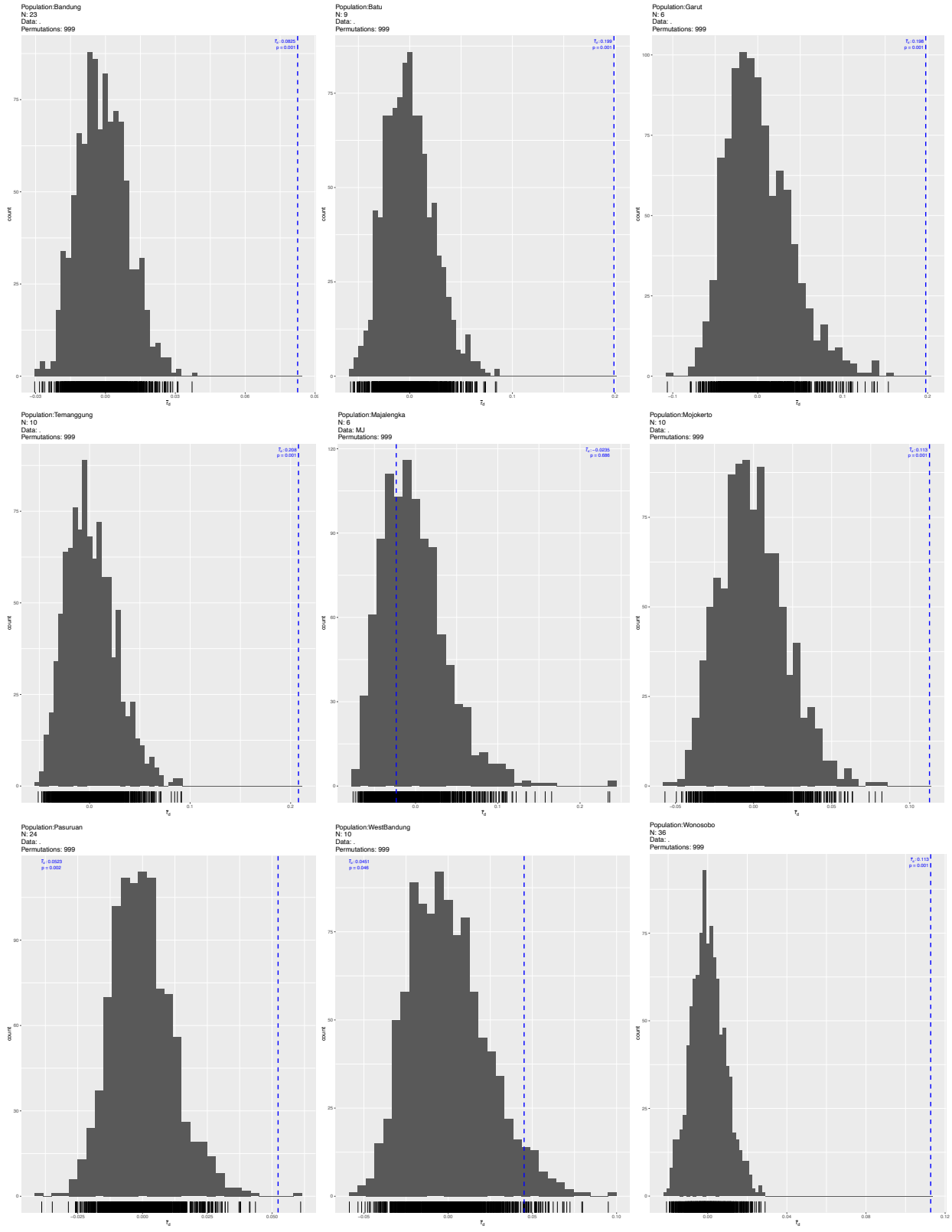
[c]eMLG, expected number of MLG for each regency. (SE, standard error)

[d]H, Shannon-Weiner Index of MLG diversity

[e]Hexp, Nei's unbiased gene diversity

[f]Ia, Index of association

[g]rbarD, Standardized index of association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Permutations: 999
Population:Batu
N: 9
Data: .
Permutations: 999
Population:Garut
N: 6
Data: .
Permutations: 999
Population:Temanggung
N: 10
Data: .
Permutations: 999
Population:Majalengka
N: 6
Data: MJ
Permutations: 999
Population:Mojokerto
N: 10
Data: .
Permutations: 999
Population:Pasuruan
N: 24
Data: .
Permutations: 999
Population:WestBandung
N: 10
Data: .
Permutations: 999
Population:Wonosobo
N: 36
Data: .
Permutations: 999
count

Figure 3.9 Mode of reproduction of *Phytophthora infestans* populations in nine regencies (clone corrected data for 12 microsatellite loci) based on the standardized index of association (rbarD). The *p*-value of rbarD (blue line) was observed in each population after clone correction. Since *p*-value is < 0.05% in all regencies except in Majalengka, we fail to reject null hypothesis which is there is no linkage among locus/markers. No linkage among locus/markers supports the evidence for clonal population in all regencies except in Majalengka (sexual reproduction).

Poppr was used to calculate the allelic diversity of the 146 DNA samples based on geographic distribution. A total of 75 different alleles were detected from 146 DNA samples ranging from 2 (SSR2) to 21 (D13) alleles, with a mean of 6.25 alleles per locus (Table 3.11). Gene diversity was estimated using Simpson's index and ranged from 0.48 (SSR2) to 0.70 (D13), indicating that the SSR2 locus was the least diverse and D13 was the most diverse locus (Table 3.11). Nei's unbiased estimation was correlated with Simpson's index. Evenness values ranged from 0.42 (D13) to 0.97 (SSR6 and SSR8). The D13 locus had the highest number of alleles (21) and high allelic diversity with the least even distribution, resulting in many MLGs (Table 3.11). PiG11 (12) and Pi4B (8) also had a high number of alleles than the rest of the loci, with higher allelic diversity and less evenness, contributing to a significant number of MLGs.

The mode of reproduction in all regencies was asexual except Majalengka (Figure 3.9). The *p*-value of rbarD of all regencies except Majalengka was smaller than 0.01. Any *p*-value greater than 0.01 is not significant and fails to reject the null hypothesis (no linkage among markers). The Majalengka population had a *p*-value greater than 0.01 for rbarD (0.235), indicating linkage among markers. Linkage among markers suggests a sexual mode of reproduction. However, more samples need to be included to get a more precise picture of the mode of reproduction.

Table 3.11 Population statistics for clone corrected microsatellite data for 12 microsatellite loci in populations of *Phytophthora infestans* from Indonesia.

Locus	**Allele**	**1-D[a]**	**Hexp[b]**	**Evenness**
D13	21	0.70	0.70	0.42
Pi4B	8	0.59	0.59	0.74
PiG11	12	0.61	0.61	0.62
Pi04	3	0.51	0.51	0.94
Pi63	5	0.66	0.66	0.89
Pi70	5	0.65	0.65	0.91
SSR2	2	0.48	0.48	0.95
SSR3	4	0.66	0.67	0.93
SSR4	6	0.66	0.67	0.83
SSR6	3	0.50	0.50	0.97
SSR8	3	0.65	0.65	0.97
SSR11	3	0.52	0.52	0.93
Mean	6.25	0.60	0.60	0.84

[a]1-D: Simpson index, [b]Hexp: Nei's 1978 gene diversity.

3.4.5 Population differentiation and structure of Bangladeshi late blight samples

Analysis of molecular variance (AMOVA) was computed based on Bruvo's genetic distance on 116 clone corrected data to determine whether variation was due to individual samples within a population or among populations. Results showed that variation between populations was only about 9 percent which is very low (Table 3.12). However, 91 percent of the variation was explained by individual samples within the population, supported by the Fst values.

The Fst calculated on clone corrected data using Bruvo's genetic distance revealed low to modest differentiation between populations (Table 3.13). The range of Fst values between populations was 0.0024 to 0.3174. The highest population differences were found between Rangpur_T and Mymensingh_P (0.3174), indicating limited gene flow between divisions or little to no migration of isolates. Mymensingh_P had the highest differentiation with other divisions; other divisions with host potato had the slightest differentiation. Rajshahi_P and Rangpur_P had the lowest population differentiation (0.0024).

Furthermore, the Fst value indicates ongoing migration events between all divisions except Mymensingh_P. The differentiation between Rangpur_P and the other divisions was the least compared to other divisions, revealing two-way migration from Rangpur_P (Table 3.13). Rajshahi_T and Rangpur_T had modest population differentiation among each other and with different populations. The DAPC analysis supports the AMOVA and pairwise Fst analysis.

Table 3.12 Analysis of molecular variance (AMOVA) for clone corrected *Phytophthora infestans* populations from Bangladesh based on Bruvo's genetic distance.

Source	**df**	**SS**	**MSS**	**% variance**
Among Populations	6	0.009	0.002	9.10
Within Populations	109	0.065	0.001	90.90
Total	115	0.074	0.0006	100

Table 3.13 Pairwise Fst for clone corrected *Phytophthora infestans* population from two host and five divisions of Bangladesh. The greater the Fst value higher the differentiation between populations. Divisions followed by P = samples collected from potato and T = samples collected from tomato.

	Sylhet_P	Mymen-singh_P	Rang-pur_P	Dhaka_P	Raj-shahi_P	Rang-pur_T	Raj-shahi_T
Sylhet_P							
Mymensingh_P	0.0151						
Rangpur_P	0.0059	0.1399					
Dhaka_P	0.0071	0.1597	0.0028				
Rajshahi_P	0.0092	0.1869	0.0024	0.0049			
Rangpur_T	0.0264	0.3174	0.0169	0.0202	0.0204		
Rajshahi_T	0.0245	0.3144	0.0188	0.0230	0.0228	0.0144	

The discriminant analysis of principal components (DAPC) results supports less variation among the divisions because most of the populations (4 out of 7) were clustered together except from three divisions (Mymensingh_P, Rangpur_T, and Rajshahi_T; Figure 3.10). All 160 samples from seven divisions were grouped in 4 clusters. Samples from Mymensingh_P, Rangpur_T, and Rajshahi_T clustered separately. However, a few of the samples from Rangpur_P overlapped with other divisions (Figure 3.10). The rest of the samples made a mixed fourth cluster.

A neighbor-joining tree (nj tree) was constructed based on Bruvo's distance with 1000 bootstraps. Most of the samples were grouped with worldwide EU_13_A2, few were grouped with EU_6_A1 and EU_1_A1, and few were grouped separately (Figure 3.11). Out of 20 samples from tomato (17 MLGs), only 3 MLGs were grouped with EU_13_A2, most of them were grouped separately, and few were grouped with EU_6_A1 and EU_1_A1. Only eight MLGs from potato were grouped separately, three grouped with EU_6_A1 and EU_1_A1, and most of them grouped with worldwide EU_13_A2. The population of *P. infestans* in Bangladesh is dominated by EU_13_A2.

A minimum spanning network was also constructed to visualize the relationship among individuals in the Bangladeshi samples and the standard genotypes of *P. infestans*. Most of the individuals from the same division were related. However, some clustered with other individuals from different divisions (Figure 3.12, 3.13, 3.14, and 3.15). Most of the individuals from Mymensingh were clustered separately from other divisions. The individuals from different divisions were scattered and found in almost every division except Mymensingh.

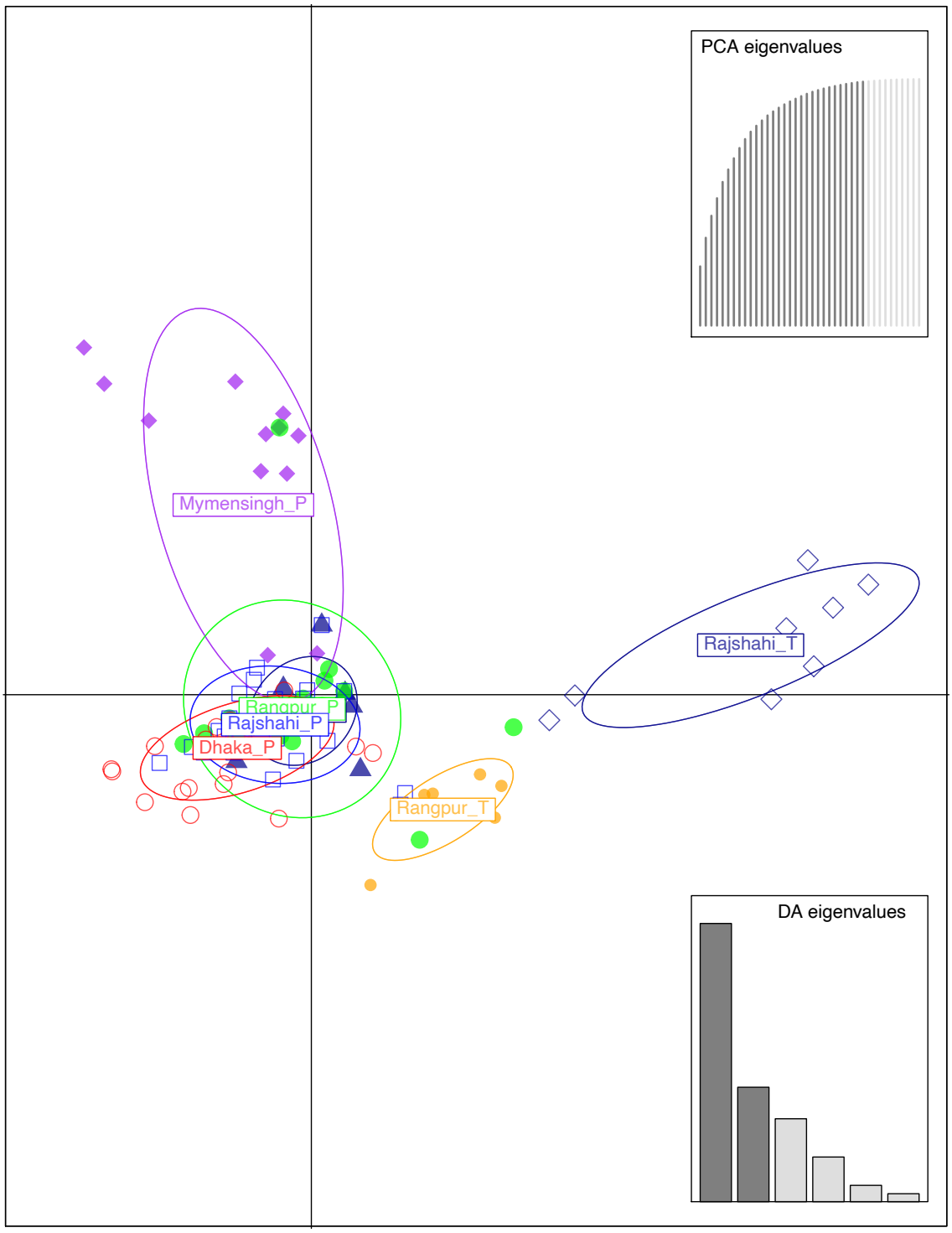


Figure 3.10 Discriminant analysis of principal components (DAPC) of *Phytophthora infestans* populations collected from five divisions of Bangladesh in 2018 (n = 94 multilocus genotypes, different letters P and T at the end of the population stands for host potato or tomato).

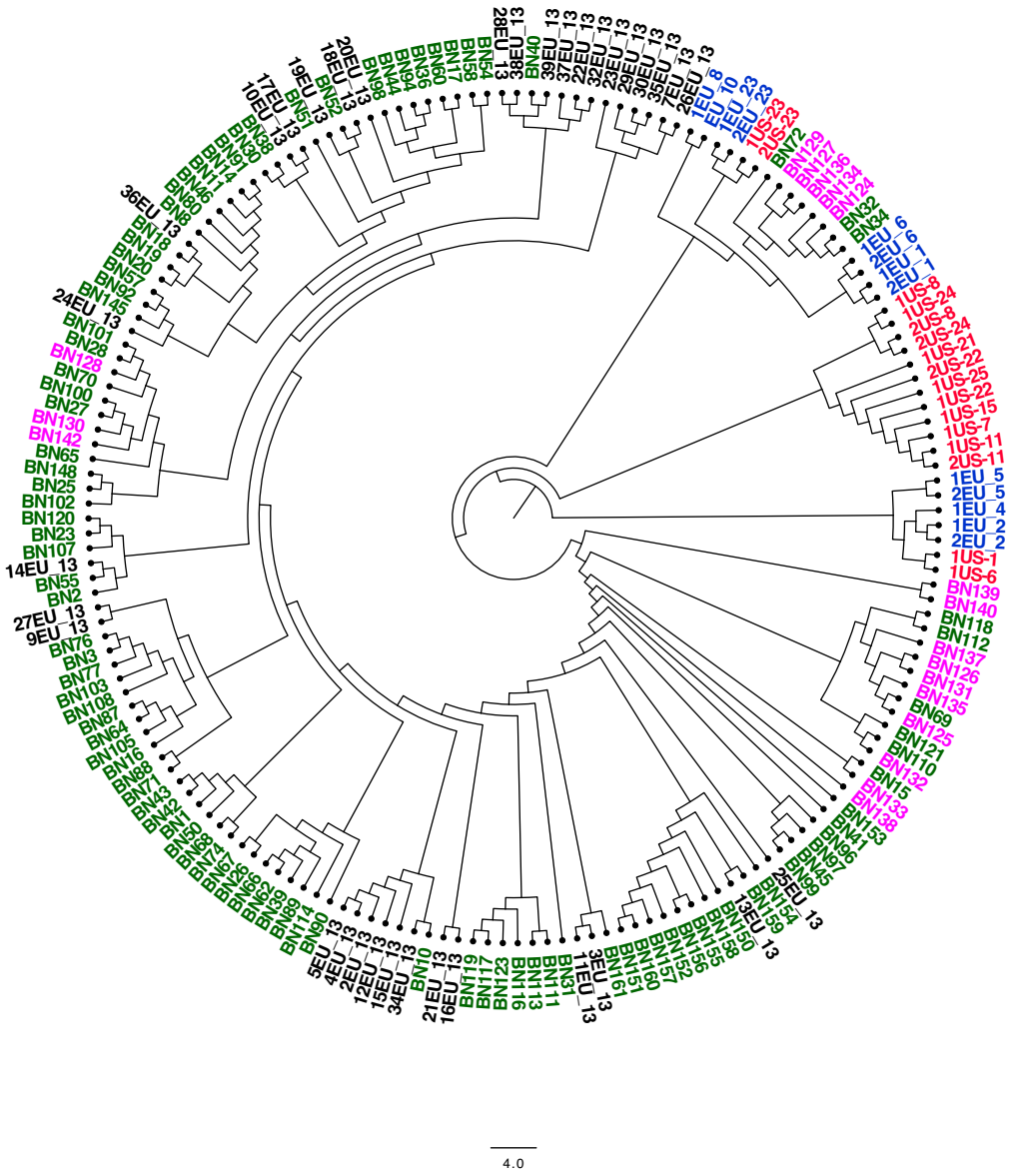


Figure 3.11 Neighbor-joining tree based on Bruvo's genetic distance for *Phytophthora infestans* populations from five divisions of Bangladesh (94 multilocus genotypes) with 1,000 bootstraps replicates. (Node labels: black● = EU_13_A2 genotype, green● = Bangladesh samples from potato, pink● = Bangladesh samples from tomato, red● = US genotypes and blue● = other European genotypes). Most of the Bangladesh samples clustered with EU_13_A2, few with EU_1_A1/6_A1 and some unique samples clustered separate showing the evidence that most of Bangladesh samples were EU_13_A2 genotypes or its variants.

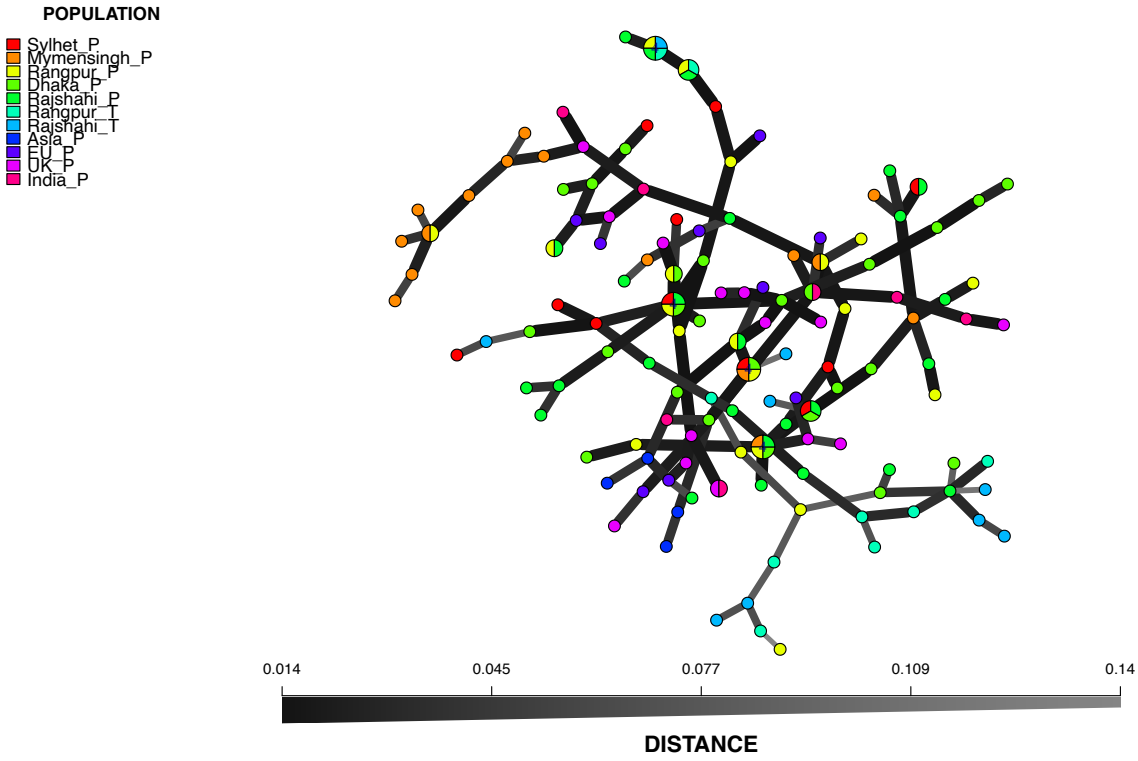


Figure 3.12 Minimum spanning network (MSN) of multilocus genotypes (MLG, n = 94) of *Phytophthora infestans* populations in Bangladesh showing the relatedness to EU_13_A2 standard MLGs from Asia, UK, Europe, and India. (Note: node size represents the size of MLGs, MLGs from Bangladesh = mixtures of MLGS from both tomato and potato. Asia, UK, and India = EU_13_A2 MLGs). The genetic distance with dark color means the MLGs are closely related, and the grey color means the MLGs are not closely related.

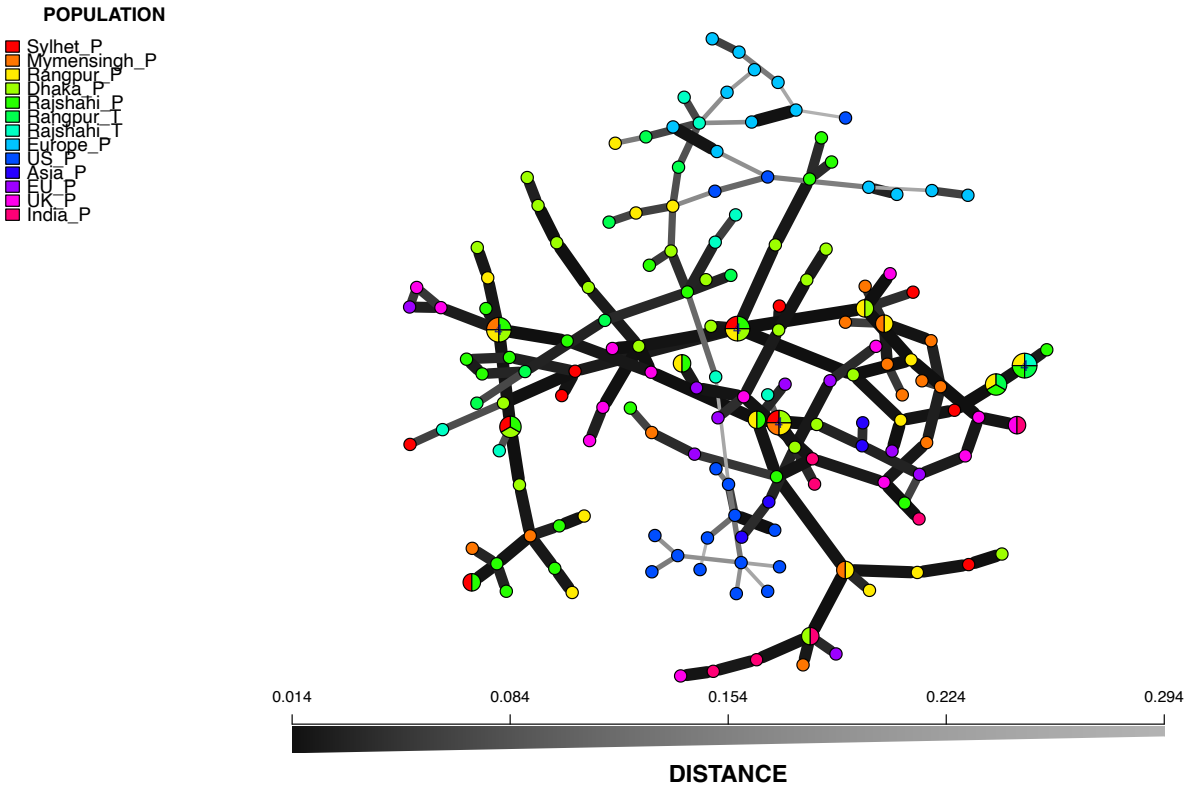


Figure 3.13 Minimum spanning network (MSN) showing the relatedness of multilocus genotypes (MLGs) of *Phytophthora infestans* populations in Bangladesh with multilocus genotypes of different clonal lineages from worldwide. (Note: the size of the node represents the number of multilocus genotypes, letter P and T in the population legend represents host tomato or potato, US_P = mixtures of different US clonal lineages, Europe_P = mixture of EU clonal lineages and EU_13_A2, Asia_P, EU_P, UK_P, and India_P = EU_13_A2). The genetic distance with dark color means the MLGs are closely related, and the grey color means the MLGs are not closely related.

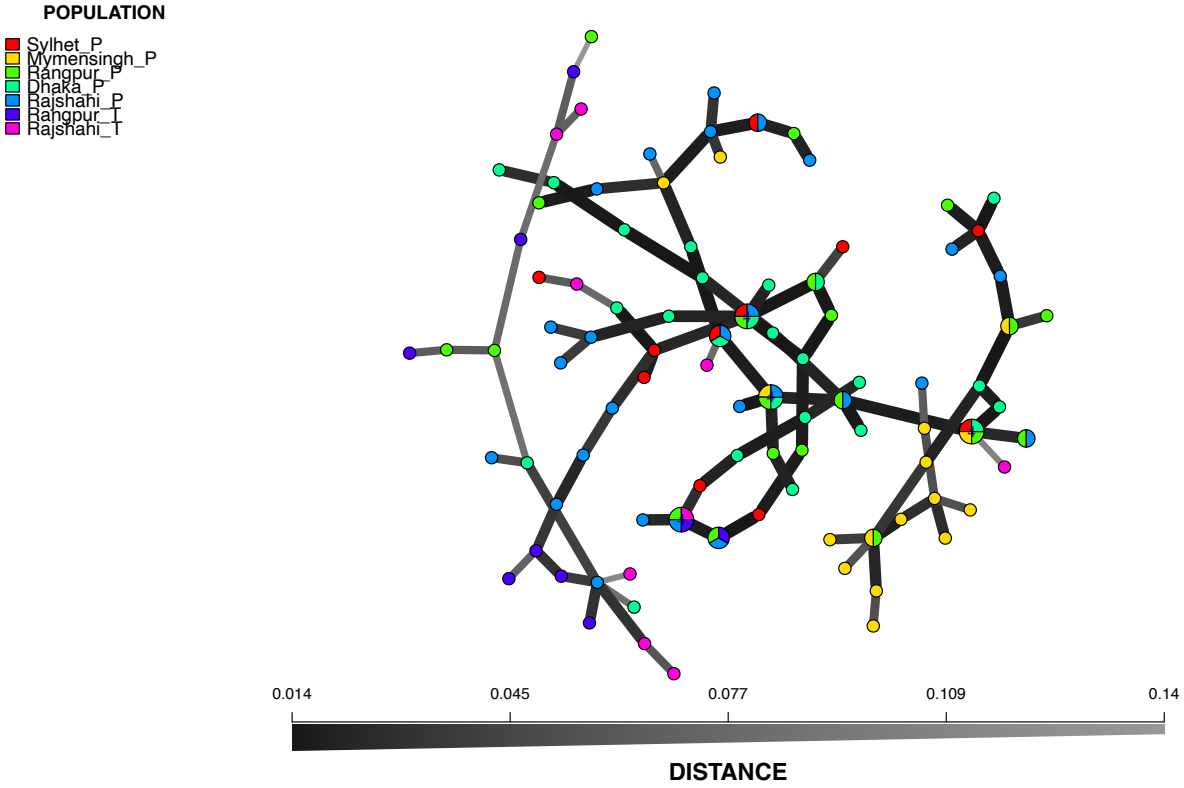


Figure 3.14 Minimum spanning network (MSN) of multilocus genotypes (MLGs, n = 94) of *Phytophthora infestans* populations in Bangladesh showing the relatedness of MLGs among each other. (Note: the size of the node represents the number of multilocus genotypes, letter P and T in the population legend represents host tomato or potato). The genetic distance with dark color means the MLGs are closely related, and the grey color means the MLGs are not closely related.

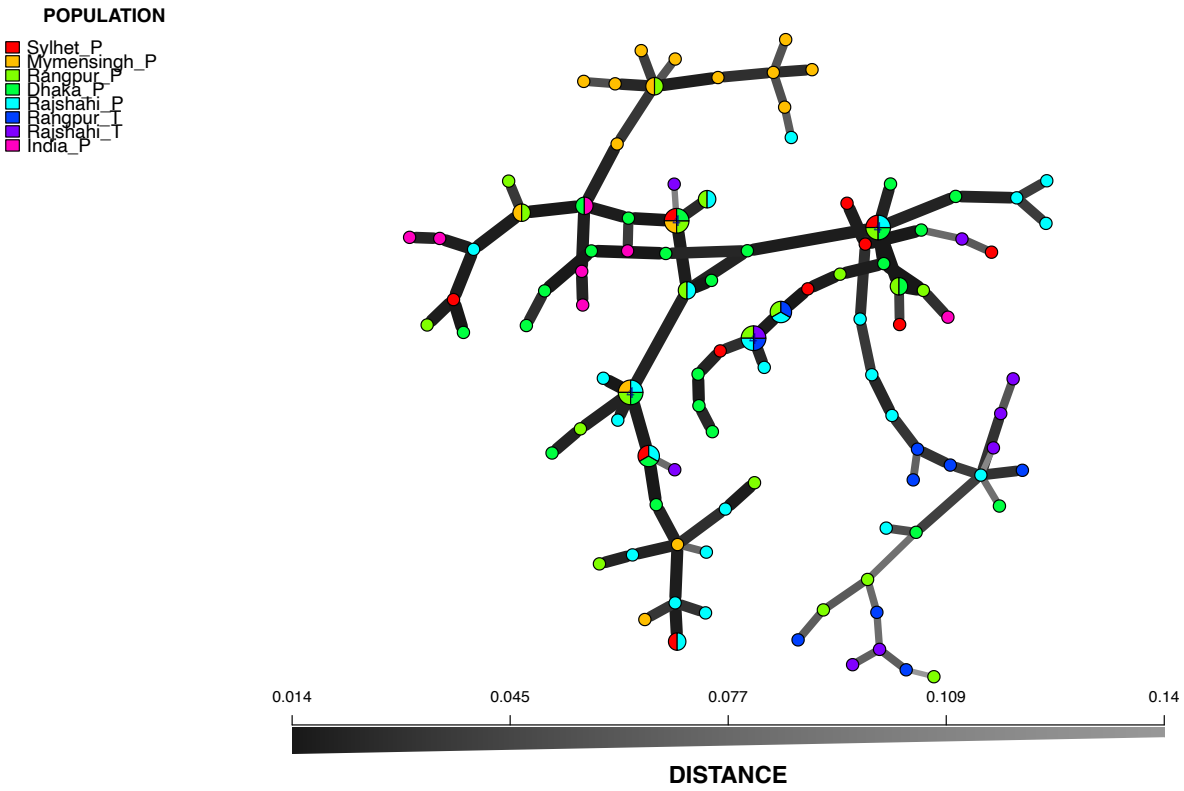


Figure 3.15 Minimum spanning network (MSN) showing the relatedness of multilocus genotypes (MLGs) of *Phytophthora infestans* populations in Bangladesh with multilocus genotypes of different variants of EU_13_A2 from India.(Note: the size of the node represents the number of multilocus genotypes, letter P and T in the population legend represents host tomato or potato, India_P = EU_13_A2).

3.4.6 Population differentiation and structure of Indonesian late blight samples

AMOVA was computed based on Bruvo's genetic distance on clone-corrected data to determine whether variation was due to individual DNA samples within a population or among populations (Table 3.14). Results showed that variation between populations (regencies) was only about 20% (Table 3.14). However, 80% of the variation was explained by individual DNA samples within the population, supported by the FST values.

The FST calculated on clone-corrected data using Bruvo's genetic distance revealed low to modest differentiation between populations (Table 3.15), with values ranging from 0.02 to 0.12. The highest population differences were found between Garut and Majalengka (0.12), indicating limited gene flow or limited migration of isolates between these regencies. Despite having the highest differentiation, these two regencies are geographically close. The lowest population differences were observed between Bandung and Wonosobo (0.02), even though these two regencies are not geographically close. Furthermore, the FST value indicates ongoing migration events between these two regencies. The lowest differentiation was between Wonosobo and the other regencies, even Majalengka, revealing two-way migration from Wonosobo, while the highest differentiation was between Majalengka and the other regencies (Table 3.15). The regencies of Wonosobo and Temanggung are geographically close and have modest differences between their populations (0.04). Likewise, Pasuruan, Mojokerto, and Batu are geographically close and have modest differentiation between their populations (0.07).

Table 3.14 Analysis of molecular variance (AMOVA) for clone corrected *Phytophthora infestans* populations based on Bruvo's genetic distance.

Source	**Df**	**SS**	**MSS**	**% variance**
Between regencies	8	3.095	0.387	19.870
Within regencies	125	10.763	0.086	80.130
Total	133	13.858	0.104	100

Table 3.15 Pairwise Fst values for clone corrected *Phytophthora infestans* population from nine regencies in Indonesia. The lower the value, the greater the similarity between the two populations.

	Bandung	West Bandung	Batu	Pasuruan	Mojokerto	Wonosobo	Garut	Majalengka	Temanggung
Bandung									
West Bandung	0.05								
Batu	0.06	0.09							
Pasuruan	0.05	0.07	0.07						
Mojokerto	0.05	0.08	0.07	0.07					
Wonosobo	0.02	0.04	0.05	0.04	0.05				
Garut	0.07	0.09	0.11	0.10	0.10	0.06			
Majalengka	0.07	0.09	0.11	0.09	0.10	0.06	0.12		
Temanggung	0.05	0.07	0.09	0.08	0.08	0.04	0.09	0.10	

The discriminant analysis of principal components (DAPC) results supports the small amount of variation among the regencies because most of the populations (6 out of 9) were clustered together except populations from three regencies (Pasuruan, Bandung, and Batu; Figure 3.16). All 146 DNA samples from the nine regencies were grouped in four clusters. DNA samples from Bandung, Batu, and Pasuruan clustered separately, while the rest clustered together to make the "mixed" fourth cluster. However, a few of the DNA samples from Wonosobo overlapped with other regencies, except Pasuruan (Figures 3.16 and 3.18).

A neighbor-joining tree (nj tree) was constructed based on Bruvo's distance with 1000 bootstraps. The NJ tree is helpful to compare our sample with the standard and already published data. Most of our samples were also grouped with standard data of EU_2_A1 (60%) EU_4_A1, and EU_13_A2 (1.5%), in an NJ tree based on Bruvo's distance (Figure 3.14). The standard US and other European genotypes clustered separately in the NJ tree.

We also constructed a minimum spanning network to visualize the relationship among individuals in the Indonesian samples and the standard genotypes of *P. infestans*. Most of the individuals were related; however, some clustered with other individuals from different regencies (Figure 3.15a and 3.15b). When some induvial are related or clustered with other individuals from different regencies, that indicates the gene flow from one regency to another or the source of inoculum for these regencies can be shared.

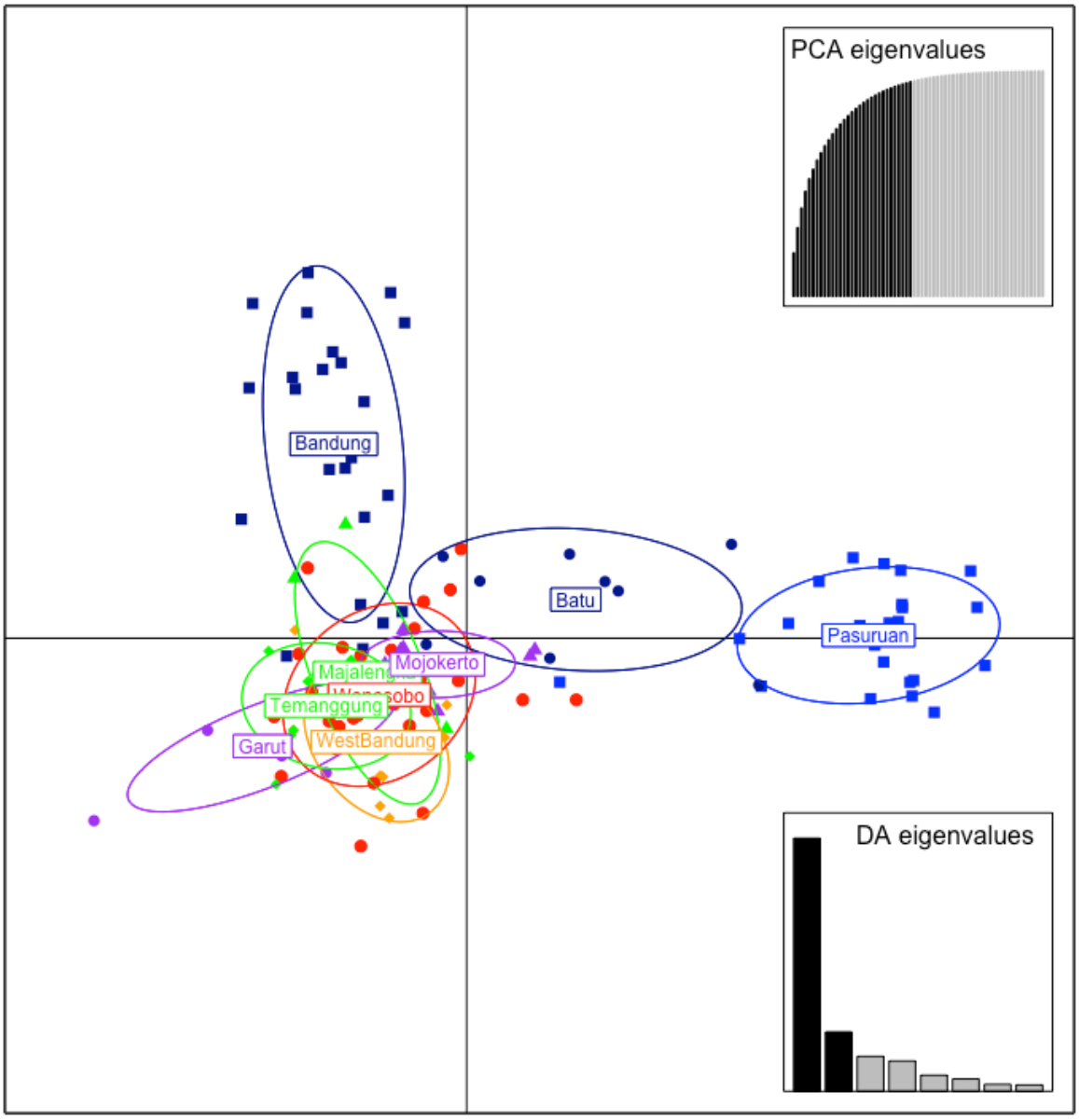


Figure 3.16 Discriminant analysis of principal components (DAPC) of *Phytophthora infestans* populations collected from 2016 to 2019 in nine regencies of Indonesia. (Data analyzed in R package poppr V.2.3.0).

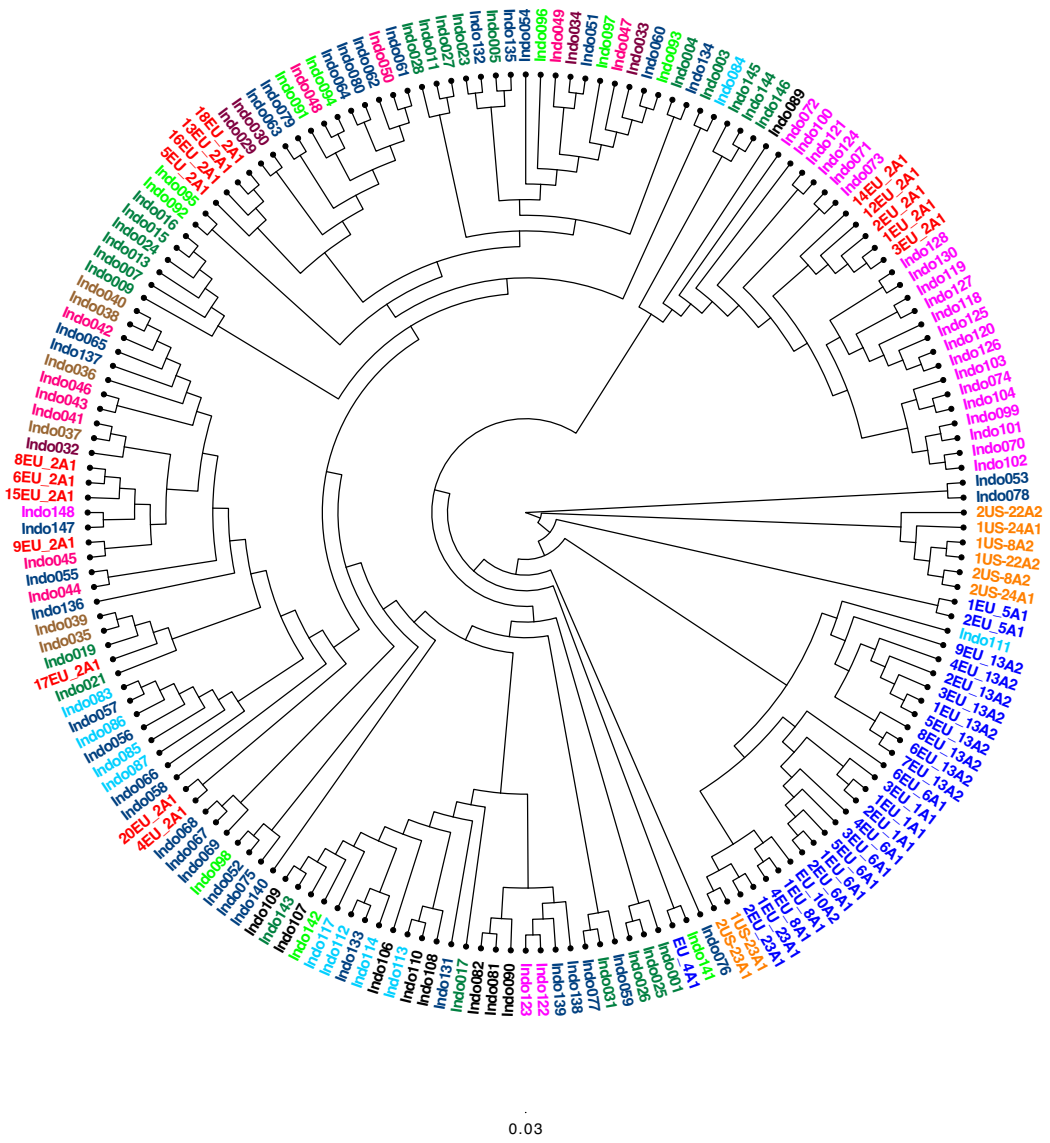


Figure 3.17 Neighbor-joining tree based on Bruvo's distance for *Phytophthora infestans* populations from nine regencies in Java, Indonesia with 1,000 bootstrap replicates. (data analyzed in the R package *poppr* V.2.3.0, results viewed and modified on Fig tree v1.4.3). Legend: Europe, Batu, Majalengka, Mojokerto, Temanggung, Pasuruan, United States, Bandung, Wonosobo, West Bandung, EU 2_A1, Garut.

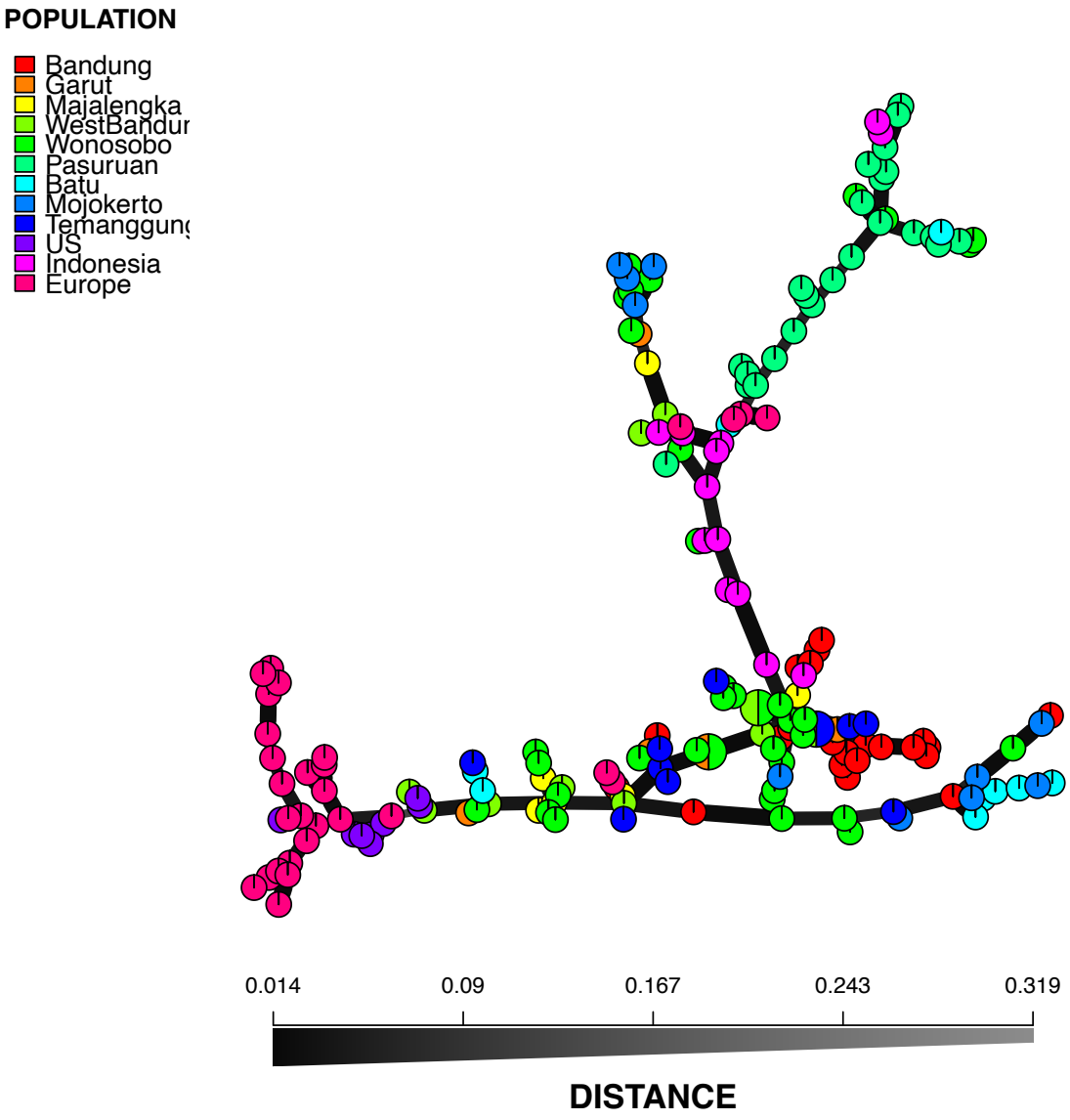


Figure 3.18 Minimum Spanning Network (MSN) of *Phytophthora infestans* isolates from Indonesia collected from 2016 to 2019 compared with some representative European and the United States isolates. Samples from Indonesia and other standard samples from Indonesia, Europe, and the USA.

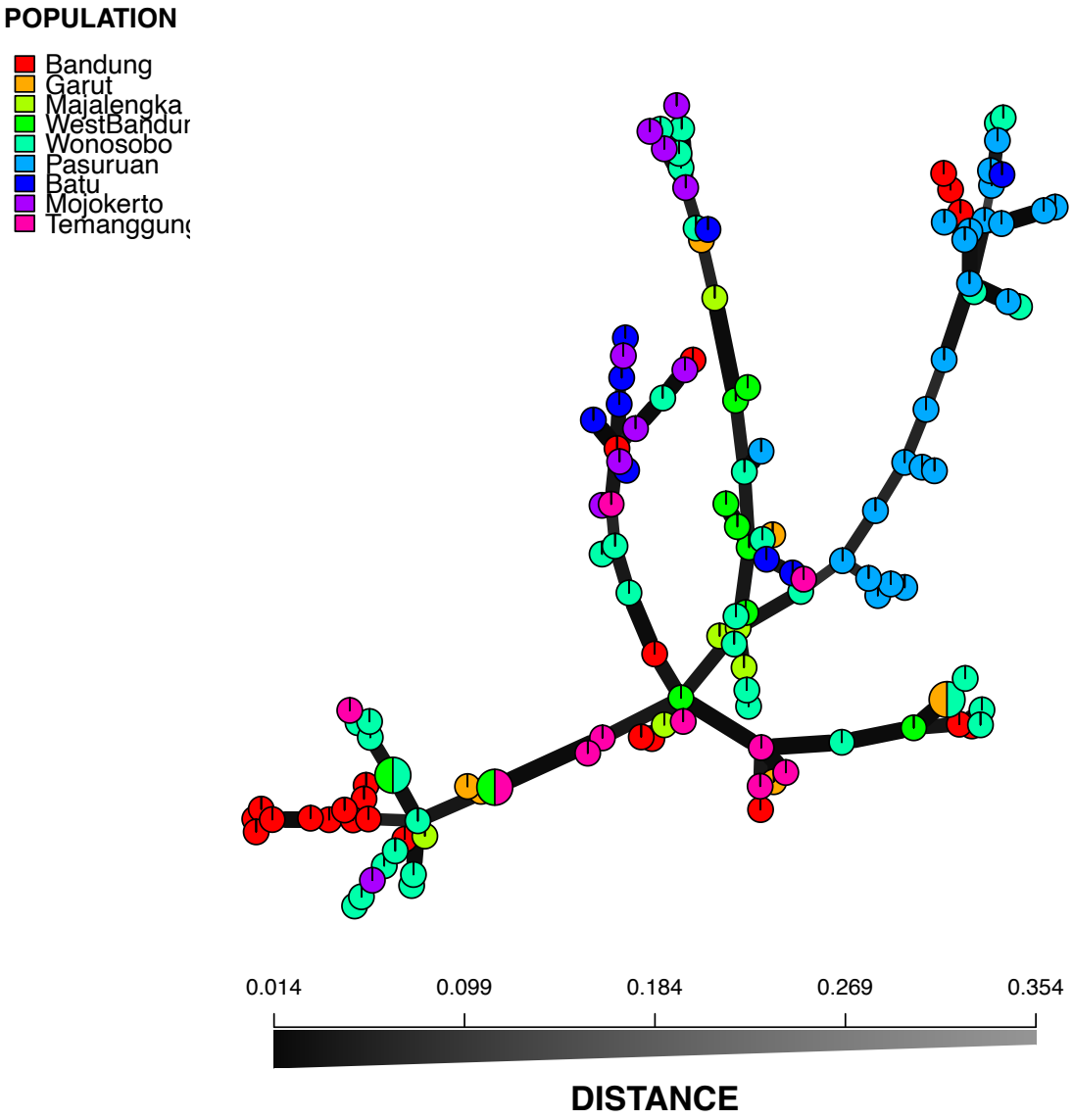


Figure 3.19 Minimum Spanning Network (MSN) of *Phytophthora infestans* isolates from Indonesia collected from 2016 to 2019 compared with some representative European and United States isolates. Samples from Indonesia only.

3.5 Discussion

3.5.1 Late blight in Bangladesh

The objective of this study was to determine the genotypes causing late blight in the main potato-growing regions of Bangladesh. Late blight has been a major impeding factor for profitable potato production in Bangladesh, where quality seed, disease forecasting systems, and fungicides with modern chemistries are unavailable to small-scale farmers. Late blight outbreaks cause severe yield loss in Bangladesh. In this study, 160 *P. infestans* samples were collected in 2018 on FTA cards and potato tubers. The SSR results of our study revealed a large sub-clonal variation and dominance of EU_13_A2 in Bangladesh. Isolates from Bangladesh were insensitive to fungicide metalaxyl-M. EU_13_A2 was supposed to have originated from The Netherlands in 2004 (Cooke et al, 2007). Previous studies on *P. infestans* population in some locations of Bangladesh reported the presence of EU_13_A2 (Asiablight; Dey et al, 2018; Pronk et al, 2017). However, these studies were done in few samples or locations. This is the first study to include multiple locations and years.

The old genotypes were reported to be diploid. The samples from Bangladesh were triploid (n=157). It was reported that clonal isolates tend to be triploid (Li et al, 2017). A large number of MLGs were detected in the *P. infestans* population of Bangladesh. 94 MLGs were found out of 160 samples. Only D13 (n=13) and PiG11 (n=7) loci had higher allelic diversity compared to other loci. Li et al (2013b) had reported a similar high diversity trend in D13 and G11 loci in EU_13_A2 strains. The standardized index of association result showed an asexual mode of reproduction in Bangladesh. Since almost all (except two A1 mating types) samples were A2 mating types, there is no or very low probability of sexual reproduction. For sexual reproduction, the population of *P. infestans* should have A1 and A2 mating types in a 1:1 ratio (Cohen et al, 1997). The pairwise differentiation analysis showed fair to low differentiation among the divisions. Rangpur_P samples were no or less different from other samples of four divisions. The low differentiation of Rangpur_P with other divisions indicates gene flow or migration event between other divisions and Rangpur. Mymensingh had the highest level of differentiation with all other divisions, which means limited gene flow from Mymensingh to other divisions or vice-versa. The AMOVA result

also supports low variation among the population. Most of the variation in the population was explained by the variation within samples.

DAPC results showed there were only four groups because most of them were clustered together. *P. infestans* population from potato were clustered together except samples from Mymensingh. The population from tomato were close but clustered separately. The samples for Rangpur_P were found everywhere. Most distinct samples were from Mymensingh_P and Rangpur_T, and Rajshahi_T as seen in DAPC and neighbor-joining tree. The differences between tomato and potato samples were evident because these two different hosts may interact with *P. infestans* strains differently, or the pathogenicity of the pathogen may differ among hosts based on the genotype of the pathogen.

The neighbor-joining tree (n=94 MLGs) indicated a large sub-clonal variation in the *P. infestans* population of Bangladesh. A similar large sub-clonal variation was found in the EU_13_A2 population in India and the EC-1 population in Ecuador (Delgado et al, 2013; Dey et al, 2018). Since a large sub-clonal variation of EU_13 A2 was found in multiple locations of India and Bangladesh, we can speculate that the environment and host in these locations are more conducive for the fitness of EU_13_A2. Since West Bengal in India and Bangladesh have similar weather conditions, EU_13_A2 may be well adapted in those locations. The significant variation in Bangladesh population may be attributed to lack of host selection, highly susceptible host, conducive environment conditions, and because potatoes are grown in neighboring countries all year round. Cooke et al (2012) had reported gene gain or loss as the primary source of variability within the clonal lineage of EU_13_A2. Since small-scale farmers grow most potatoes in Bangladesh, they might not have access to or could not afford modern fungicide chemistries. Because of that, some farmers might well spray their fields, whereas other farmers may not. It has been reported that *P. infestans* tend to lose alleles when exposed to the fungicides (Li et al, 2017). In contrast, higher genetic diversity was observed in the *P. infestans* population, which were not exposed to metalaxyl-M fungicides (Grunwald et al, 2006). This can be one of the reasons for the large sub-clonal variation in the *P. infestans* population of Bangladesh.

The samples collected from tomatoes were unique, and most of them were grouped together in the neighbor-joining tree. Only three samples clustered together with standard EU

13_ A2. EU_13_A2 was reported to be pathogenic on tomatoes (Chowdappa et al, 2013). The difference in susceptibility or different cultivars of potato and tomato might have impacted the clonal diversity of EU_13_A2. Some sub-clonal isolates of EU_13_A2 were reported to infect only tomatoes or are less aggressive on potatoes (Dey et al, 2018). Almost all samples tested with the different the DNA markers were determined to be A2 mating types except two A1 mating types from Rangpur_P. The samples collected from Mymensingh were also unique compared to other samples. However, these samples (n=12 out of 16 MLGs) were clustered together with the single UK isolate, suggesting these isolates might have been introduced from the UK. In this study, samples collected from tomato (n=5) and potato (n=3) were closer to EU_6_A1 and EU_1_A1, but they were A2 mating types based on CAPS and other markers. However, these markers cannot entirely (100%) assign the isolates/samples to specific mating types (Brylińska et al, 2018). To be sure about the mating types, isolates should be collected, and mating types should be determined using a pairing test. Six out of eight samples clustered together with EU_6_A1 and EU_1_A1 were from the Rangpur division (tomato n=3, potato n=3).

The genotypes of *P. infestans* have been widely spread from the center of origin through infected seed tubers. The genotypes migrating to new locations might dominate the existing genotypes depending upon their fitness in those locations. Recently, EU_13_A2 has been reported to be prevalent in some countries of Asia such as India, Pakistan, Nepal, China, Myanmar, and Bangladesh (Chowdappa et al, 2013, 2015; Dey et al, 2018; Cooke et al, 2019). EU_13_A2 changed the population structure of the *P. infestans* in these countries. Chowdappa et al (2013) reported the introduction of EU_13_A2 in India, which caused a severe outbreak of late blight in tomatoes in 2009. Chowdappa et al (2013) speculate that the EU_13_A2 was introduced to India through seed potatoes imported from the UK or The Netherland. The same genotype caused a severe outbreak in India in 2015 and kept dominating the *P. infestans* population with increased sub-clonal diversity (Chowdappa et al, 2013; Dey et al, 2018). Since neighboring countries like Nepal, India, and Myanmar have EU_13_A2, the genotype could have easily crossed the border in rainstorms or wind (Chowdappa et al, 2013, 2015; Dey et al, 2018; Cooke et al, 2019). Bangladesh produces a wide variety of potatoes, including Granola which was imported from The Netherlands. As

EU_13_A2 was found in The Netherlands in 2005, it could have been introduced in Bangladesh through the infected asymptomatic seed potatoes (Cooke et al, 2007).

The metalaxyl sensitivity test results showed that EU_13_A2 is insensitive to metalaxyl-M at 100 µg/L. EU_13_A2 is very pathogenic, and growers in Bangladesh could not control late blight even with multiple fungicide sprays. In some locations, sporulation was seen in recently sprayed fields showing no response to the fungicides applied. Since A1 mating types samples were also found in Bangladesh, and if the ratio of A1 and A2 mating types reach 1:1, there will be the possibility of sexual reproduction, which can change the population structure of *P. infestans*. However, it is necessary to collect more samples from infected tomato plants because the samples collected from infected tomatoes were different than EU_13_A2 but closer to EU_6_A1.

3.5.2 Late blight in Indonesia

This study provides the first in-depth investigation into the genotypes that make up the *P. infestans* populations in the main potato growing regions on the island of Java, Indonesia. Although in the past a few studies have been conducted with limited isolates, populations of *P. infestans* in Indonesia have not been thoroughly studied until now. Nishimura et al (1999) included four samples from an unknown location in Indonesia in 1993 while characterizing the *P. infestans* population in seven Asian countries. They found that all four were A2 mating types and resistant to metalaxyl-M. Our study only found one DNA sample, Indo 111, that was identified as A2 using CAPS markers. However, this mating type could not be confirmed using the Phyb markers. All our DNA samples that were successfully amplified were determined to be the A1 mating type based on the CAPS marker results, and these results were corroborated with the pairing test carried out with six isolates from Indonesia. Brylińska et al (2018) have reported that isolates were not perfectly (100%) assigned to specific mating types using these molecular markers (CAPS and Phyb assigning isolates correctly by 96% and 84%, respectively). As such, Brylińska et al (2018) recommended that local populations such as these need to be validated with the pairing test method before these markers are used for DNA samples.

Populations of *P. infestans* in Indonesia were reported to be resistant to metalaxyl-M (Nishimura et al, 1999; Adiyoga, 2009). However, of the six isolates we tested for metalaxyl-

M sensitivity, two were found to be intermediate, and four were found to be sensitive to metalaxyl-M (Table 3.7). With our small sample size, it is impossible to determine how widespread resistance to metalaxyl- M is on Java. However, due to the genetic diversity of *P. infestans* isolates from Java, it is probable that there are isolates with a range of metalaxyl-M sensitivities on the island, depending on the local use of metalaxyl-M.

Microsatellite analysis revealed that most of our samples were EU_2_A1 or subclonal variants of EU_2_A1 based on the SSR fingerprints compared with already published data, our standards, and data kindly provided by Dr. David E. L. Cooke (Li et al, 2013; David Cooke, The James Hutton Institute, Invergowrie, Dundee, UK, personal communication). Dr. Louise Cooke also reported that the limited number of samples obtained from West Java as part of an AsiaBlight study were all EU_2_A1 (Queen's University, Belfast, UK, personal communication). However, we found large subclonal variation in the Indonesian *P. infestans* population.

Based on the SSR fingerprints and NJ tree analysis, about 60% of the population were EU_2_A1 with a large sub-clonal variation. There are many reasons behind the large variation within clonal lineages. One is polyploidization, which increases the number of alleles in a locus (Li et al, 2017). In our analysis, there were DNA samples that were diploid, triploid, and tetraploid. Most of our DNA samples were triploid (n = 131). Of 12 loci, six were diploid, five were triploid, and a few DNA samples at D13 loci were tetraploid (Figure 3.4). In addition to that, some loci had a higher number of alleles with high genetic diversity and less evenness, such as D13 and PiG11. The number of MLGs we found was increased due to polyploidization in alleles in six loci. It has been reported that clonal lineages tend to have more alleles (triploids) in multiple loci than the progeny of sexual recombinants (Li et al, 2013). The occurrence of polyploidization may mask accumulated deleterious alleles in asexual populations (Li et al, 2017) that otherwise may lead to extinction during evolution, according to Muller's law (Muller, 1964). That is why there is a prevalence of clonal lineages worldwide, as they are so successful due to their adaptability and fitness under many environmental conditions, even without sexual recombination (Li et al, 2017). We also found clonal reproduction based on the linkage disequilibrium analysis (conducted with both raw and clone-corrected data) except in Majalengka. However, we had a small number of samples from this regency, and we needed to include more samples to get a more precise result.

Shakya et al (2018) found a different trend; most of the *P. infestans* isolates in Mexico had diploid alleles in sexual and clonal populations. However, Li et al (2017) reported that most successful clonal lineages tend to be triploid because they have more heterozygous single nucleotide polymorphisms (SNPs) and a higher level of functional variations compared to diploids.

Another reason for sub-clonal variation may be a mutation. Indonesia is right under the equatorial line and is subjected to high UV levels, resulting in a higher mutation rate, as described in Ecuador (Delgado et al, 2013). Similar large sub-clonal variations of single clonal lineages were observed in Ecuador and India (Delgado et al, 2013; Dey et al, 2018). Large variations have also been reported in Nordic European countries (Brurberg et al, 2011). However, the variation in Nordic European countries is mainly due to sexual recombination (Brurberg et al, 2011). Due to the subtropical climate, multiple potato crops can be grown throughout the year in Indonesia, India, and Ecuador, so the disease cycle is maintained year-round. Li et al (2017) stated that *P. infestans* isolates tend to decrease their number of alleles under adverse climatic conditions such as low carbon or exposure to sublethal levels of metalaxyl-M fungicide. In Indonesia, growers use fungicide sprays extensively, with up to 30 sprays in a single season (Adiyoga, 2009). Excessive use of fungicides may impact polyploidization (Li et al, 2017). However, factors such as high UV radiation, a continuous year-round disease cycle, small-scale growers who may not spray fungicides extensively, continued use of susceptible cultivars, and very conducive environmental conditions for late blight in Java might lead to different scenarios where polyploidization levels are not impacted or are even increased. From our results, we can speculate that the populations of *P. infestans* on the island of Java, Indonesia, are undergoing mutation through allele loss or gain.

The distribution of genetic variation in Java populations was examined using AMOVA. Individual samples within the regencies contributed 80% of the variation compared to the populations between regencies that explained only 20%. We determined a similar trend in population differentiation. FST values, which ranged from 0.02 to 0.12, indicated that the differentiation between populations was low to modest. In recent years, potato seed tubers have been locally produced in West Java, with few seeds imported from Germany and the Netherlands (Fugile et al, 2006; Adiyoga, 2009). The population differentiation analysis revealed evidence of higher migration between regencies of West Java

and Central Java, such as between Wonosobo/Bandung, Wonosobo/West Bandung, and Wonosobo/Temanggung than between West Java and East Java. However, we did observe similar levels of population differentiation between Wonosobo in Central Java and Pasuruan in East Java, suggesting there has been migration between populations in Central and East Java.

Genetic diversity was found to be highest in Garut, but in Majalengka, it was found to be lower compared to other regencies. The discrepancy in genetic diversity from these regencies is most probably due to the limited number of samples collected. Still, it could also be due to a lack of migration or gene flow between these regions. The population differentiation based on FST values also supports low or lack of gene flow in these two regencies. Linkage disequilibrium analysis of our DNA samples suggests that the mode of reproduction in *P. infestans* populations from Majalengka is sexual (Figure 3.9). However, five were determined to be A1 mating types from this regency out of six DNA samples. So, the question arises as to how it can be possible to have sexual recombination without the A2 mating type? Because the number of DNA samples we collected from this region was so small, it is most likely that there are A2 mating types in the area. Nishimura et al (1999) reported the presence of A2 mating type isolates from Java, so it is probable that there are A2 mating types in the population that were not collected in our samples. Majalengka was the only regency in Java with a population where we detected indications of sexual reproduction. More DNA samples and isolates need to be collected over multiple years to get a clearer view of this regency's primary mode of reproduction.

In the NJ tree based on Bruvo's genetic distance, we found that 62% of our DNA samples clustered with European isolates. However, 38% of DNA samples clustered separately from the European isolates, while one sample clustered with the US isolates. Some of the *P. infestans* populations in regencies such as Batu and Bandung are dominated by unique MLGs. The Batu population was composed of all unique MLGs. The Bandung population was mixed with a few EU_2_A1 (based on SSR fingerprints matched with published data) and unique MLGs. In contrast, the Wonosobo population was found to have a mixture of sub-clonal variants of EU_2_A1 and a few unique MLGs. The Pasuruan population was mainly dominated by EU_2_A1, which was also supported by DAPC analysis.

We also found 1.5% of the samples recovered were EU_4_A1 or EU_13_A2. EU_2_A1 and EU_4_A1 are older European genotypes dominant in Europe over 20 years ago, and EU_13_A2 was first identified in isolates obtained from the Netherlands in 2004. Martin et al (2019) classified worldwide haplotypes of *P. infestans* and discovered these European genotypes all had the same haplotype (I-15 of Ia). The I-15 of Ia haplotype contributed this as maternal parents for other contemporary genotypes. Because Indonesia imported Granola seed tubers from Europe, these genotypes may have been introduced to Java in infected symptomless seed tubers. The European genotypes most probably served as maternal parents on Java from which unique genotypes could have evolved. With the history of the A2 mating type and resistance to metalaxyl-M, the EU_13_A2 clonal lineage in Mojekerto (in one DNA sample from the current study) potential sexual reproduction Majalengka, close monitoring, and intensive sampling are necessary for these regencies.

This study provides insights into the structure and diversity of *P. infestans* populations in Java, Indonesia. There have been many reports of late blight epidemics caused by older lineages of *P. infestans* and their increasing prevalence in different parts of the world (Njoroge et al, 2019a). In east Africa, the US-1 clonal lineage seemingly dominated *P. infestans* populations since its introduction in Kenya in 1941 (Njoroge et al, 2019a). However, the US-1 lineage has recently been completely displaced in Kenya by EU_2_A1and, almost completely replaced in other east African countries (Njoroge et al., 2019a). The increased dominance of EU_2_A1 in east Africa has been attributed to its increased aggressiveness on potatoes (Njoroge et al, 2019b). We discovered EU_2_A1 dominating *P. infestans* populations on Java, particularly in the East Javan regency of Pasuruan, which clustered separately from West and Central Javan regencies in the DAPC analysis. EU_2_A1 may be the original genotype introduced to Indonesia. It is now being displaced in Central and West Java by variants of EU_2_A1 and unique MLGs identified in these regions.

In Europe, programs to monitor and track changes in populations of *P. infestans* over time have been used to enhance the effectiveness of current management strategies. These strategies have included host resistance management, fungicide program optimization, the use of integrated pest management tools, and sophisticated decision support systems (Cooke et al, 2011). In Indonesia, these systems do not exist, and growers rely almost exclusively on

fungicides to produce viable yields of potatoes. With our studies showing isolates of *P. infestans* with intermediate sensitivity to metalaxyl-M, total reliance on fungicides to manage late blight is not the best option. Alternative sources of disease management such as resistant cultivars should be given consideration. One such alternative would be the development of bioengineered local varieties with 3 R-gene resistance to late blight, such as the ones being developed by the USAID Feed the Future Biotechnology Potato Partnership (FtFBPP). This would be of significant benefit to potato growers in Indonesia because it would reduce the cost of production through fewer fungicide costs, reduce farmer exposure to fungicides, and reduce fungicide residues in food, land, and wastewater.

Before and after releasing resistant cultivars in late blight-prone areas, it is crucial to track the diversity of *P. infestans* genotypes over time, monitoring for factors or new isolates that may overcome resistance. Tracking of genotypic diversity of *P. infestans* is critical to ensure the effectiveness and durability of any 3 R-gene cultivars released in Indonesia. The data from this study will serve as a baseline to inform the development of integrated strategies to extend the efficacy and durability of the USAID FtFBPP 3 R-gene potato cultivars developed for release in Indonesia.

4 DEVELOPMENT OF A DIAGNOSTIC ASSAY FOR RAPID ON-SITE DETECTION OF *PHYTOPHTHORA INFESTANS*

4.1 Summary

Early detection, coupled with knowledge of the genotype present, can ensure the timely implementation of the most optimum disease management strategy. Recently, loop-mediated isothermal amplification (LAMP) assays have become more widely used for the rapid on-site detection of *P. infestans,* but these assays have limitations. This study developed a new LAMP assay using the *ypt1* gene for *P. infestans*. Our assay can easily distinguish *P. infestans* from other oomycetes such as *Phytophthora erythroseptica, P. mirabilis, P. nicotianae, and Pythium ultimum* within 10 minutes on a Genie II or IIIC platform. In addition, six other published LAMP assays were compared with our assay on Genie IIIC using the same concentration of primers. Results showed that our assay was more reliable than other assays based on specificity and sensitivity on the Genie platform. Our LAMP assay based on the *ypt1* gene did not cross-react with *P. mirabilis* or *P. phaseoli*. However, although our assay did cross-react with *P. andina* and *P. ipomoeae*, *P. ipomoeae* was easy to distinguish because it amplified very late in the reaction. The lower limit of detection (LOD) of our LAMP assay was determined to be 1 pg/µL (LAMP run for 25 min) for pure culture. LAMP and quick DNA extraction technology, coupled with a portable platform such as the Genie IIIC, enable the rapid on-site detection of *P. infestans*. Samples confirmed as *P. infestans* can then be characterized further into different genotypes in the lab settings.

4.2 Introduction

Numerous diagnostic assays have been developed to detect and diagnose pathogens based on culture or nucleic acid amplification using time-consuming and labor-intensive methods. Commonly used PCR or qPCR requires different temperature settings for denaturation, annealing, and extension of target DNA. Thus, PCR requires rapid heating and cooling steps while the reaction is running. In general, most PCR instruments have large heating and cooling elements, making them too heavy and not portable enough to be used

outside a lab. Many real-time PCR assays have been developed using a different sequence that can detect *Phytophthora infestans* from various sources with high sensitivity. However, these assays were lab-based, and some cross-reacted with close relatives of *P. infestans* in clade 1c (Atallah et al, 2006; Böhm et al, 1999; Lees et al, 2012; Llorente et al, 2010). A few field-based diagnostic tools have been developed that are suitable for on-site detection of pathogens, such as pocket diagnostic, lateral flow devices (LFDs), and immunostrips. However, these are not species-specific or cross-reacts with close relatives of the target pathogen (Abingdon Health, Sand Hutton UK; Agdia, Elkhart IN USA; Avila et al, 2009; Martin et al, 2012; Tomlinson et al, 2010b).

Many DNA-based diagnostics tools have been developed to detect *P. infestans* on different platforms (Hansen et al, 2016; Khan et al, 2017; Lees et al, 2012, 2019; Ristaino et al, 2019). These LAMP-based diagnostic assays have been developed to detect pathogens rapidly and accurately from different sources. However, some LAMP assays have serious limitations, mainly due to the specificity and sensitivity of the primers used in the assay. The latest incarnations of these instruments, the Genie II, III, and IIICc, are like qPCR machines in that the reaction is monitored using fluorescent probes, and results are displayed on a color LCD screen on the device. However, being battery-powered and small enough to fit in a camera backpack, these machines are ideally suited for in-field diagnostics.

In terms of specificity, cross-contamination or false positive is crucial while developing an on-site diagnostics assay. There may be species of *Phytophthora* present in potato fields that are not pathogenic to potatoes. The detection of these secondary *Phytophthora* species may be misidentified as *P. infestans*. Hence, the primary aim of this study was to develop a sensitive and very species-specific LAMP assay that did not cross-react with non-pathogenic species of *Phytophthora* that may be present in a potato field. Thus, the objectives of this study were to a) develop a *P. infestans* specific and sensitive LAMP assay, b) compare our LAMP assay with similar ones that have already been published, and c) determine the limit of detection of *P. infestans* in naturally infected field samples using our LAMP assay.

4.3 Materials and methods

4.3.1 Plant material and oomycete isolates used in the study

Leaves from potato plants (cv. Russet Burbank) infected with late blight were received from growers in Idaho. *Phytophthora infestans* isolated from the leaves were identified as the genotype US-23. Isolates were characterized using a mating type test, isozyme analysis, and molecular markers according to the methods described in Chapter 3 (Dangi et al, 2021). A total of 13 samples, six genotypes of *Phytophthora infestans*, six close relatives of *P. infestans* and *Pythium ultimum* were used in the study (Table 4.1). Cultures of *P. infestans* were maintained on pea agar (Dangi et al, 2021), whereas *P. erythroseptica* and *Pythium ultimum* were maintained on potato dextrose agar, and *P. nicotianae* on V-8 media. DNA from other closely related species of *Phytophthora*, such as *P. mirabilis*, *P. andina*, *P. ipomoeae*, and *P. phaseoli,* was obtained from the World Phytophthora Genetic Resource Collection (University of California, Riverside).

We also inoculated leaves of cultivar Russet Burbank in the lab. Disease-free leaves from 6-week-old plants were selected for inoculation. For inoculation, leaves were placed abaxial surface up on a wire grid in a rectangular glass container lined with a moist paper towel. The wire grid elevated the leaves off the moist paper towel. A sporangial suspension was prepared from 14-day old *P. infestans* cultures. About 2 mL of sterile distilled water was added, and the mycelia were scraped gently with an L-shaped glass rod or scalpel. The suspension was incubated at 4ºC for 4 hours to encourage the release of zoospores. Leaves were inoculated with 10 µl droplets of a zoosporangial suspension of *P. infestans* placed in the center of the leaf. The lid was then placed on the container, and the containers were placed in an incubator at 18ºC for 3-5 days. Infected leaves were used for various experiments, including DNA extraction using different methods.

4.3.2 DNA extraction using polyethylene glycol buffer

Phytophthora infestans DNA was extracted from sporulating lesions on infected leaves using the quick DNA extraction method described by Chomczynski and Rymaszewski (2006). The DNA extraction method uses a high alkaline lysis reagent (polyethylene glycol and potassium hydroxide) for quick extraction without further DNA isolation steps. The PEG buffer was prepared as previously described by Chomczynski and Rymaszewski (2006). Briefly, 60 g of a 60% PEG 200 solution (Sigma-Aldrich, St. Louis, MO, USA) was mixed with 0.93 ml of 2 M potassium hydroxide (KOH) in 39 mL of water. The pH was measured and adjusted (if necessary) by adding extra KOH to bring the final pH to 13.3. A single sporulating lesion from an infected leaf was cut into small pieces and added (0.1 – 0.5 g) to a 5 mL Eppendorf tube containing 1 mL of PEG buffer and a steel bearing ball. The tissue was macerated by shaking the tube. The sample was finely macerated the tube was allowed to rest for a couple of minutes, so all the particulate matter sank to the bottom. A micropipette was then used to transfer 20 µL aliquot of the suspension (avoiding particulates) to fresh tubes containing 180 µL of water. The diluted original concentration by ten-fold brought the pH down to approximately 7. After maceration, samples could be kept in the freezer for later use or used the right way in LAMP and qPCR assays.

Genomic DNA from cultures and infected leaves was extracted using modified Cetyl Trimethyl Ammonium Bromide (CTAB) buffer and a magnetic bead in a robotic Kingfisher mL (Thermo Scientific, UK) as described in Chapter 2.

4.3.3 Primer design

The ras-related *ypt1* gene of *P. infestans* was selected (accession no. KU720615.1) to design the species-specific LAMP primers. Multiple sequence alignments of the *ypt1* gene of species closely related to *P. infestans* were examined with the alignment program to identify the species-specific primer sequences using Primer Express 2.0 (Applied Biosystems, Warrington, UK). Three sets of primers were developed: forward and backward primers (F3 and B3), two internal primers (FIP, F2-F1c, and BIP, B2-B1c), consisting of two segments of sequence from a different location. The F2/B2 sequence segment was between the F3/B3 primers and F-loop/B-loop primers (Fig 4.1). An assay was designed to confirm that none of the primers cross-reacted with *Phytophthora* species closely related to *P. infestans*. In

addition, a set of stem primers were developed, which included the loop segment of the sequence to speed up the reaction. Details of the LAMP assay sequence and length of each primer sequence are listed in Table 4.1 and Figure 4.1.

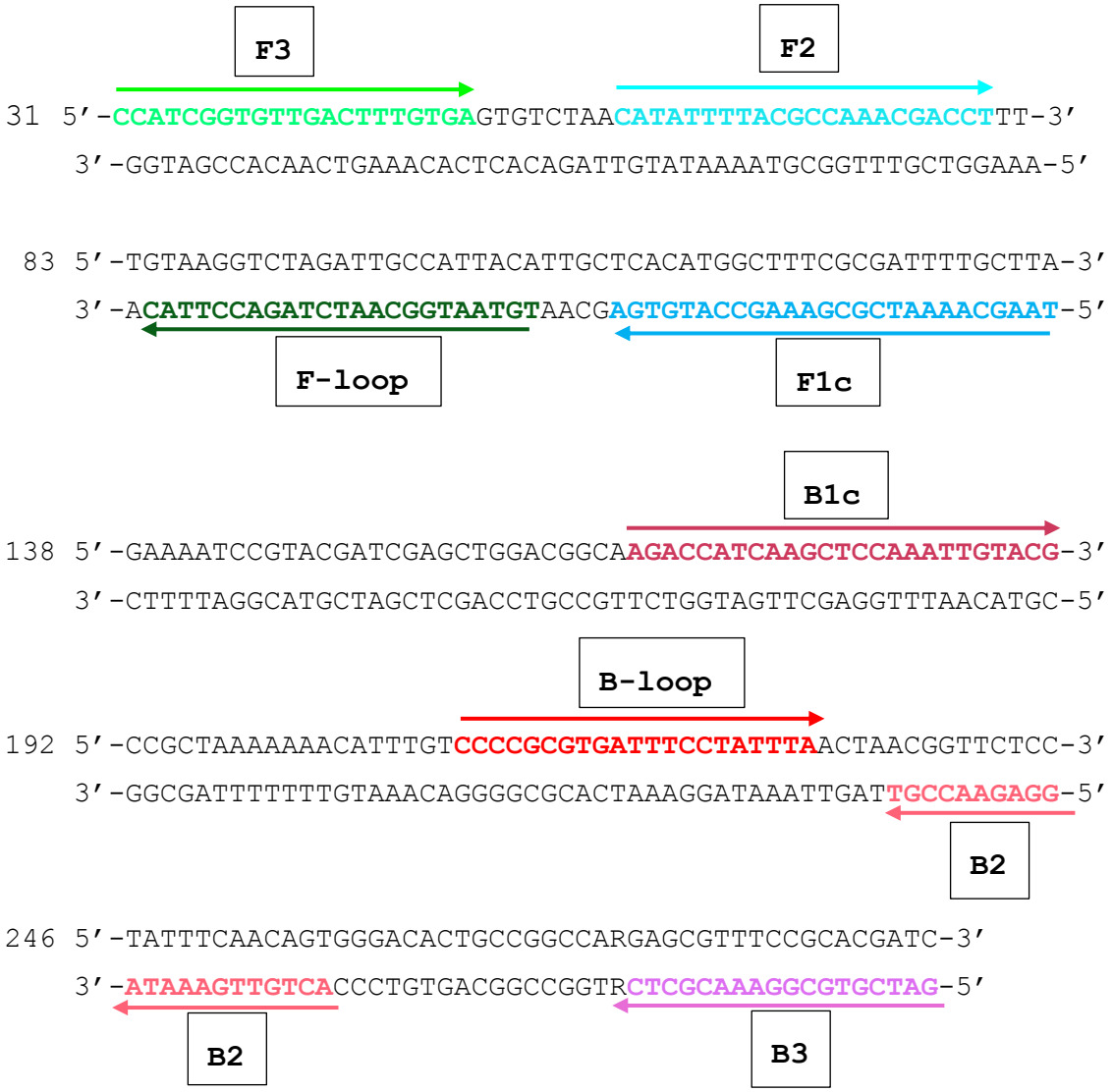


Figure 4.1 Location and sequence of primers of *Phytophthora infestans* on the selected 262 bp target sequence of the ras-related *ypt1* gene. (FIP = F1c-F2 and BIP = B1c-B2).

Table 4.1 Details of the primers and probes sequences from the ras-related *ypt1* gene developed for this study.

Primer/Probe	Sequence
F3	5’- CCATCGGTGTTGACTTTGTGA-3’
B3	5’- GATCGTGCGGAAACGCTC-3’
FIP	5’-TAAGCAAAATCGCGAAAGCCATGTGACATA
	TTTTACGCCAAACGACCT-3’
	F1c 5’-TAAGCAAAATCGCGAAAGCCATGTGA-3’
	F2 5’-CATATTTTACGCCAAACGACCT-3’
BIP	5’-AGACCATCAAGCTCCAAATTGTACGACTGT
	TGAAATAGGAGAACCGT-3’
	B1c 5’-AGACCATCAAGCTCCAAATTGTACG-3’
	B2 5’-ACTGTTGAAATAGGAGAACCGT-3’
F-loop	5’-TGTAATGGCAATCTAGACCTTAC-3’
B-loop	5’-CCCCGCGTGATTTCCTATTTA-3’
Stem 1	5’ -CAGAAAATTCGCACGATCGA-3’
Stem 2	5’ -ATTCGCACGATCGAGCTG-3’

4.3.4 Comparison of loop-mediated isothermal amplification (LAMP) assays

The new LAMP assay developed in the study was compared with already published assays (Table 4.2) to compare the performance, specificity, and sensitivity.

Optigene isothermal master mix N001 (Optigene) was compared with newer Optigene isothermal master mix N004 in our LAMP assay to see if there were any differences in time to positive (tp) value. Stem primers were also developed and tested against both master mixes and the three primer pairs developed and used in the study. These stem primers target the "Stem region" in the LAMP amplicon, hence named Stem primers (Gandelman et al, 2011).

Table 4.2 Comparison of the current and previously published assays used for the detection of *Phytophthora infestans*.

Assay	Chemistry	Gene Target	Cycling conditions	Amplicon size	Specificity (cross-reaction)	Limit of detection	Time to positive/ Ct value	Ref.
Si Ammour	Fluorescence	ITSII	Isothermal (65°C)	255 bp	*P. andina*, *P. mirabilis*, *P. phaseoli, P. ipomoeae*	50 fg/µL	11-13 min	Si Ammour et al. (2017)
Hansen ITS	Color change	ITSII	Isothermal (65°C)	226 bp	nt*; *P. nicotianae*	2 pg/µL	1 hr	Hansen et al. (2016)
Hansen Rgn86_2	Color change	Rgn86_2	Isothermal (65°C)	169 bp	*P. andina* *P. mirabilis*	200 pg/µL	1 hr	Hansen et al. (2016)
Khan	Color change	*ypt1*	Isothermal (65°C)	205 bp	nt	0.128 pg/µL	30-70 min	Khan et al. (2017)
Lees	Fluorescence	ITSI and ITSII	Isothermal (65°C)	252 bp	*P. ipomoeae*, *P. mirabilis*, *P. phaseoli*, *P. palmivora*	5 pg/µL	40 min	Lees et al. (2019)
Ristaino	Color change	ITS	Isothermal (65°C)	220 bp	nt	1 pg/µL	35 min	Ristaino et al. (2019)
Current study	Fluorescence	*ypt1*	Isothermal (65°C)	262 bp	*P. andina* *P. ipomoeae*	1 pg/µL	8 min	This study
Atallah	qPCR SYBR	*ras*	qPCR	121 bp	nt, *P. mirabilis*, *P. phaseoli*	nt	19.9 min	Atallah (2006)
Khan SYBR	qPCR SYBR	*ypt1*	qPCR	83 bp	nt	12.8 pg/µL	~ 20 min	Khan et al. (2017)
Llorente	qPCR SYBR	Sequence repeat	qPCR	-	nt	2 pg/µL	~ 20 min	Llorente et al. (2010)

Böhm	qPCR TaqMan	Nr satellite DNA	qPCR	73 bp	nt	1fg/µL	~19 min	Böhm et al. (1999)
Lees TaqMan	qPCR TaqMan	ITS	qPCR	167 bp	*P. mirabilis*, *P. phaseoli*, *P. ipomoeae*	100 fg/µL	32.2 min	Lees et al. (2012)

nt*: not tested in the original paper for sensitivity or specificity with close relatives of *P. infestans* in clade 1c (*P. andina*, *P. mirabilis*, *P. phaseoli, P. ipomoeae*)

Note: Some Assays did not test all the species of close relatives of *P. infestans* in clade 1c.

4.3.5 LAMP reactions and amplification conditions

All LAMP reactions were performed in custom 8-well strips of tubes made to fit the reaction block of the Genie II and IIIC with a final volume of 25 µL: 15 µL of isothermal master mix N004 containing fluorescent dye (Optigene, UK), 3 µL of template DNA and varying concentrations of primers and water depending on different assays being studied. The experiments were carried out on a Genie II or Genie IIIc using the default LAMP condition for 25 minutes (Optigene). Two isothermal master mixes from Optigen Ltd. N001 and the new N004 were compared to determine assay performance differences.

4.3.6 Sensitivity and specificity

Our LAMP assay and six other previously published assays (Table 4.2) were tested for cross-reaction (exclusivity test) with species of *Phytophthora* that are closely related to *P. infestans.* These included *P. mirabilis*, *P. ipomoeae*, *P. andina*, *P. phaseoli*, *P. nicotianae*, *P. erythroseptica,* and closely related species *Pythium ultimum* which causes a common potato disease in the USA. For the inclusivity test, six (four from the USA and two from the UK) different genotypes of *P. infestans* were investigated. A series of 10-fold dilutions were prepared from pure culture DNA (US-23 genotype) for the sensitivity test, ranging from 1 ng/ µL to 1 fg/ µL. Only one genotype was selected to determine the limit of detection of the assays because all six genotypes had a close time to positive value. The sensitivity test was also conducted with DNA extracted from infected leaves with CTAB and purified using the Promega magnetic bead purification kit and Kingfisher mL or the cruder PEG buffer rapid extraction method. Five series of 10-fold dilutions were prepared and tested. For the specificity test, the experiments were repeated twice, and if there was significant variation between them, they were repeated a third time. Experiments were repeated three times for all the assays for the sensitivity tests. The DNA used in these experiments was from either the World Phytophthora Genetic Resource Collection (University of California, Riverside) or standard DNA from our lab (pure cultures characterized using standard characterization methods).

4.4 Results

4.4.1 Specificity test of different assays

All seven LAMP and five qPCR assays were tested with various genotypes of *P. infestans*, relative species of *P. infestans,* and *Pythium ultimum* (Table 4.3-4.6). Lees assay failed to detect any standard *P. infestans* genotypes under default LAMP conditions on the Optigene II and IIIc, so it was excluded from further study.

4.4.1.1 Inclusivity test of different assays

Our assay and all the previously published assays detected genotypes of *P. infestans* (Fig 4.2; Table 4.3 and 4.4). However, each assay gave a different time to positive in min (tp value) for each isolate. The Amour assay detected *P. infestans* genotypes the fastest (tp approximately 4 min), and the Khan LAMP assay detected *P. infestans* genotypes the slowest (tp ~ 22 min) compared to the other assays (Table 4.3). Our LAMP assay detected *P. infestans* genotypes at around tp 7-8 min depending on the genotypes of *P. infestans*. Khan LAMP assay exceeded 20 min in detecting any genotypes of *P. infestans* used in this study.

Table 4.3 Loop-mediated isothermal amplification (LAMP) assay performance – inclusivity testing with a pure culture of different *Phytophthora infestans* genotypes (Tp: time to positive in minutes and seconds).

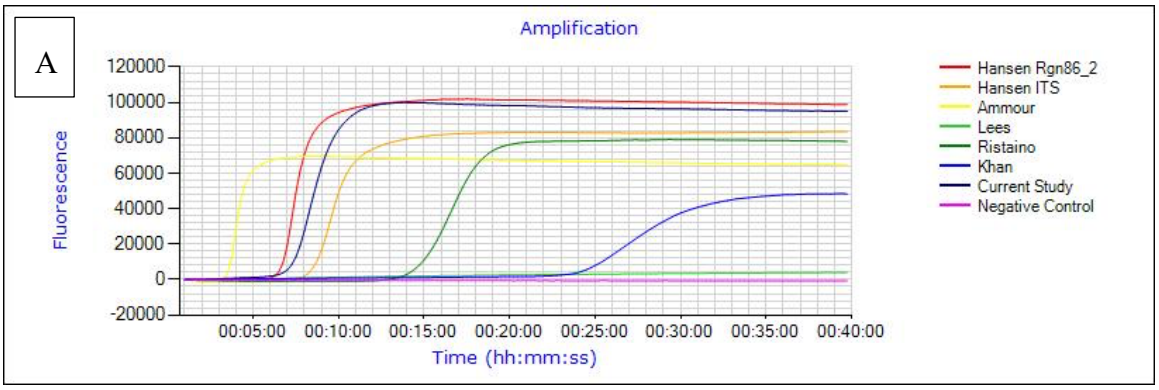
Genotypes (Isolates)	Si Ammour (Tp)	Hansen (ITS, Tp)	Hansen (Rgn86_2, Tp)	Khan (Tp)	Ristaino (Tp)	Current study (Tp)
US-8 (A2)	4.15	7.23	6.0	23.24	12.30	7.65
US-22 (A2)	3.38	6.15	5.38	20.73	12.30	7.00
US-23 (A1)	4.16	9.81	7.66	24.09	17.08	8.08
US-24 (A1)	4.45	8.38	8.58	23.58	18.58	9.45
EU 13_A2	4.08	6.65	5.73	24.16	15.38	8.08
EU 6_A1	4.23	7.38	7.08	-	17.08	8.73

Table 4.4 Quantitative real-time PCR (qPCR) assay performance – inclusivity testing with a pure culture of different *Phytophthora infestans* genotypes (CT: threshold cycle value).

Isolates	Atallah (Sybr, Ct value)	Khan (Sybr, Ct value)	Llorente (Sybr, Ct value)	Böhm (TaqMan Ct value)	Lees (TaqMan Ct value)
US-8 (A2)	35.3	28.0	28.4	24.2	23.7
US-22 (A2)	32.1	25.8	26.9	26.3	19.3
US-23 (A1)	35.1	29.6	26.3	24.0	23.3
US-24 (A1)	36.1	28.9	29.5	22.9	23.2
EU 13_A2	30.5	25.5	26.2	20.1	19.1
EU 6_A1	35.1	28.7	24.2	22.1	21.8

Note: Any Ct value > 0 is positive or confirms the assay cross-reacts with that isolate, whereas Ct 0 is negative, and the assays do not cross-react with those isolates.

A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80000
60000
40000
20000
0
-20000
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00:15:00
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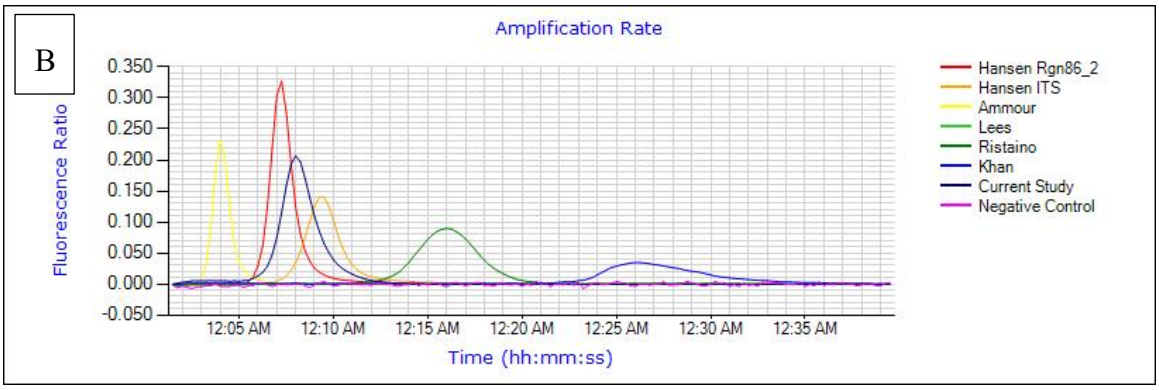
00:30:00
00:35:00
00:40:00
Time (hh:mm:ss)
Hansen Rgn86_2
Hansen ITS
Ammour
Lees
Ristaino
Khan
Current Study
Negative Control

B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0.200
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0.100
0.050
0.000
-0.050
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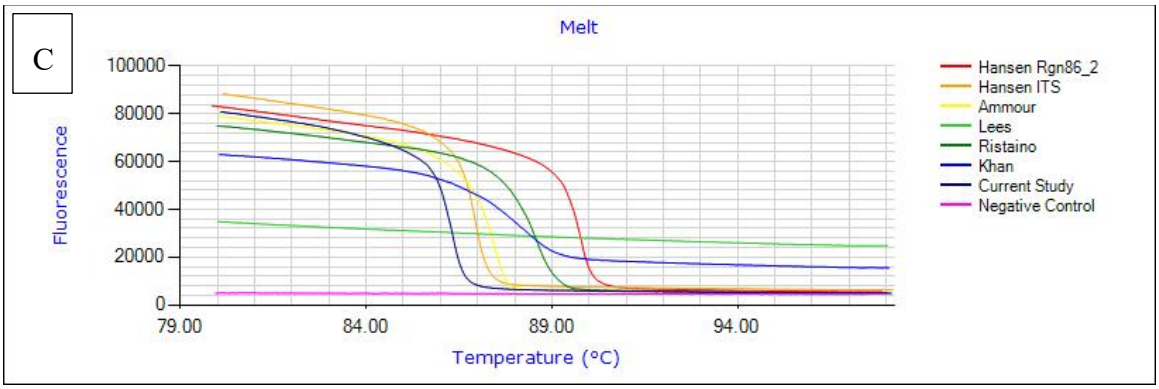
12:10 AM
12:15 AM
12:20 AM
12:25 AM
12:30 AM
12:35 AM
Time (hh:mm:ss)
Hansen Rgn86_2
Hansen ITS
Ammour
Lees
Ristaino
Khan
Current Study
Negative Control

C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Fluorescence
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40000
20000
0
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84.00
89.00
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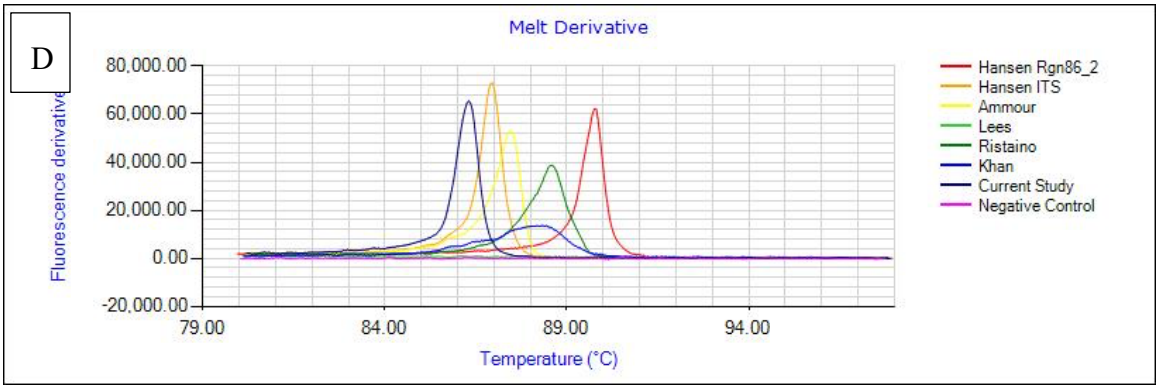
Temperature (°C)
Hansen Rgn86_2
Hansen ITS
Ammour
Lees
Ristaino
Khan
Current Study
Negative Control

D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Fluorescence derivative
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40,000.00
20,000.00
0.00
-20,000.00
79.00
84.00
89.00
94.00
Temperature (°C)
Hansen Rgn86_2
Hansen ITS
Ammour
Lees
Ristaino
Khan
Current Study
Negative Control

Figure 4.2 A. Amplification curve, B. amplification rate, C. melt temperature, and D. melting curve of different LAMP assays compared in this study. The sample used in this test was US-23 genotypes of *Phytophthora infestans*. (Current study: assay developed in this study). The seven assays had different time to positive starting from ~ 4 min to 22 min (A), amplification rate (B) and annealing temperature ranging from 84ºC to 91ºC. Khan assay did not amplify.

4.4.1.2 Exclusivity test of different assays

All the assays detected the genotypes of *P. infestans,* and some cross-reacted with closely related species of *Phytophthora* (Table 4.5 and Table 4.6). Ammour's LAMP Assay cross-reacted with the species closely related to *P. infestans* (clade 1c) described by Si Ammour et al (2017). However, this assay had the lowest time to positive (tp) value for all genotypes of *P. infestans*. The Hansen's ITS-based LAMP assay cross-reacted with all the species of *Phytophthora,* including *P. nicotianae* and *P. erythroseptica*. The Hansen's Rg86_2 LAMP assay cross-reacted with *P. andina*, *P. mirabilis,* and *P. ipomoeae*. However, *P. ipomoeae* was detected very late in the reaction. Khan's LAMP assay detected *P. infestans* genotypes very late (tp ~22) but did not cross-react with any other *Phytophthora* species. The Ristaino's LAMP assay cross-reacted with all species closely related to *P. infestans.* Our assay only cross-reacted with *P. andina* and *P. ipomoeae*. However, *P. ipomoeae* was detected very late in the reaction with a tp ~18 min (Fig 4.3), while *P. infestans* was detected early at tp 7-8 min (Fig 4.2).

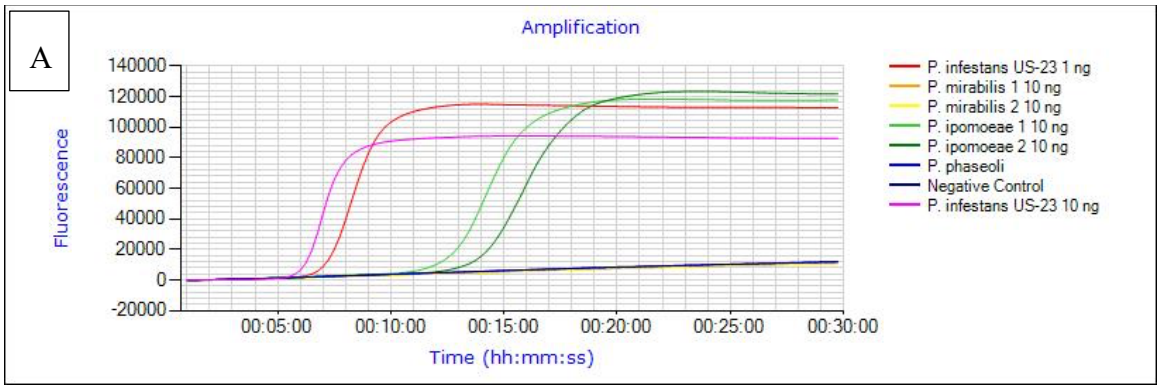
All TaqMan and Sybr assays cross-reacted with *P. andina* (Table 4.6). The Atallah's Sybr assay detected *P. phaseoli*, *P. mirabilis* and *P. mirabilis* very late in the reaction (Ct ~39-45). This could be avoided by lowering the qPCR cycle to 40. The Khan's Sybr assay detected *P. mirabilis* and *P. ipomoeae*. The Llorente's Sybr assay detected all species in clade 1c (*P. mirabilis*, *P. andina*, *P. ipomoeae*, and *P. phaseoli*). The Bohm's TaqMan assay also detected all species on clade 1c except *P. ipomoeae*. The Lees TaqMan assay detected all species in clade 1c and *P. erythroseptica*.

Table 4.5 Loop-mediated Isothermal Amplification (LAMP) assay performance – exclusivity testing with a pure culture of different *Phytophthora* species and *Pythium ultimum* (Tp: time to positive).

Isolates	Si Ammour (Tp)	Hansen (ITS, Tp)	Hansen (Rg86_2, Tp)	Khan (Tp)	Ristaino (Tp)	Current study (Tp)
Phytophthora mirabilis	4.45	9.23	8.15	-	17.73	-
Phytophthora andina	4.45	9.23	7.95	-	18.88	9.31
Phytophthora ipomoeae	4.88	9.23	21.73	-	20.31	18.24
Phytophthora phaseoli	7.30	9.80	-	-	19.73	-
Phytophthora nicotianae	-	13.44	-	-	21.30	-
Phytophthora erythroseptica	-	10.61	-	-	-	-
Pythium ultimum	-	-	-	-	-	-

Table 4.6 Quantitative real-time PCR (qPCR) assay performance –exclusivity testing with a pure culture of different *Phytophthora* species and *Pythium ultimum* (CT: threshold cycle value).

Isolates	Atallah (Sybr, Ct value)	Khan (Sybr, Ct value)	Llorente (Sybr, Ct value)	Böhm (TaqMan Ct value)	Lees (TaqMan Ct value)
Phytophthora mirabilis	39.9	27.7	25.5	25.7	21.3
Phytophthora andina	32.3	26.9	27.8	20.8	20.3
Phytophthora ipomoeae	-	26.8	23.0	-	17.6
Phytophthora phaseoli	40.7	-	30.9	26.6	23.3
Phytophthora nicotianae	44.5	-	-	-	-
Phytophthora erythroseptica	-	-	-	33.1	35.4
Pythium ultimum	-	-	-	-	-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Amplification
Fluorescence
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120000
100000
80000
60000
40000
20000
0
-20000
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00:10:00
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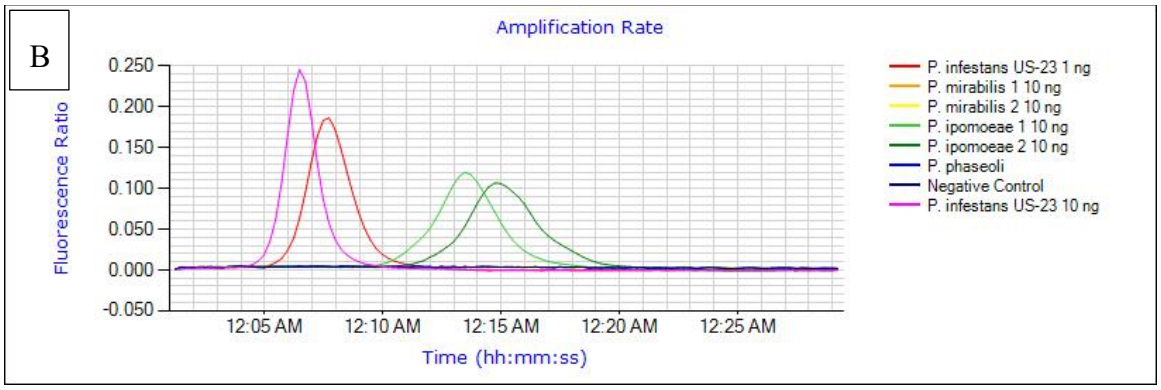
00:20:00
00:25:00
00:30:00
Time (hh:mm:ss)
P. infestans US-23 1 ng
P. mirabilis 1 10 ng
P. mirabilis 2 10 ng
P. ipomoeae 1 10 ng
P. ipomoeae 2 10 ng
P. phaseoli
Negative Control
P. infestans US-23 10 ng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Fluorescence Ratio
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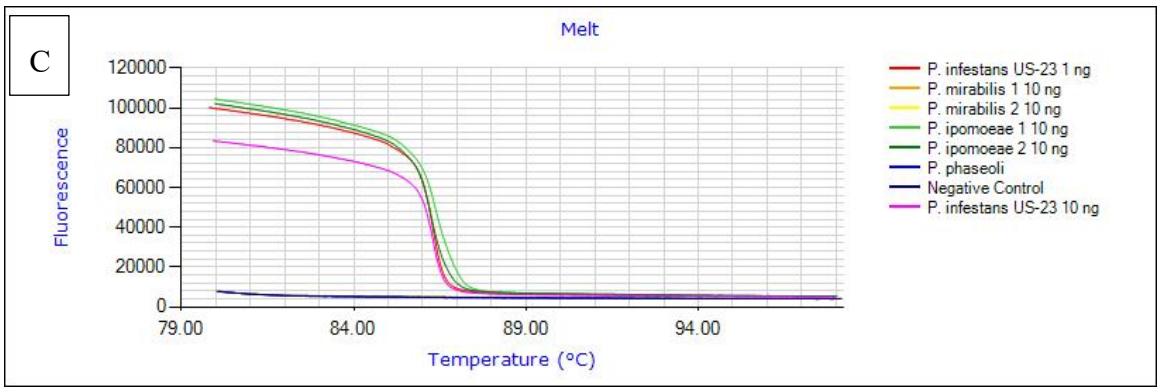
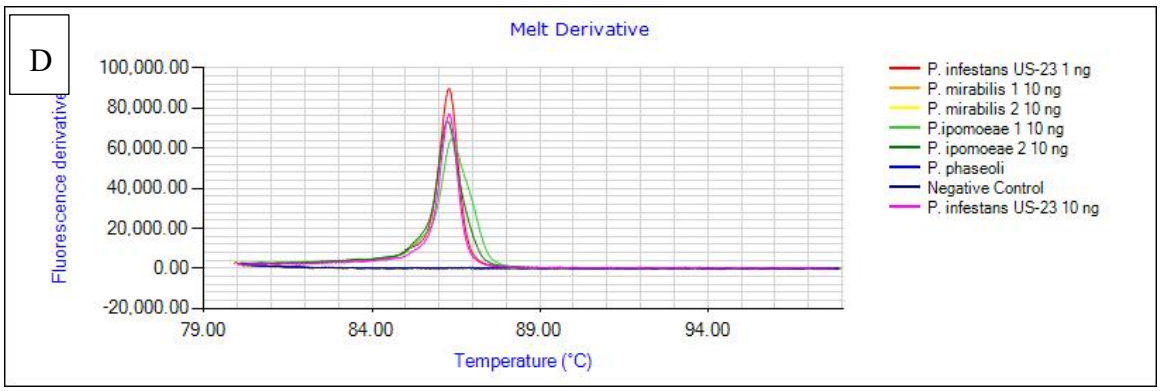
-0.050
12:05 AM
12:10 AM
12:15 AM
12:20 AM
12:25 AM
Time (hh:mm:ss)
P. infestans US-23 1 ng
P. mirabilis 1 10 ng
P. mirabilis 2 10 ng
P. ipomoeae 1 10 ng
P. ipomoeae 2 10 ng
P. phaseoli
Negative Control
P. infestans US-23 10 ng


C
Melt
Fluorescence
120000
100000
80000
60000
40000
20000
0
79.00
84.00
89.00
94.00
Temperature (°C)
P. infestans US-23 1 ng
P. mirabilis 1 10 ng
P. mirabilis 2 10 ng
P. ipomoeae 1 10 ng
P. ipomoeae 2 10 ng
P. phaseoli
Negative Control
P. infestans US-23 10 ng


D
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Fluorescence derivative
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40,000.00
20,000.00
0.00
-20,000.00
79.00
84.00
89.00
94.00
Temperature (°C)
P. infestans US-23 1 ng
P. mirabilis 1 10 ng
P. mirabilis 2 10 ng
P.ipomoeae 1 10 ng
P. ipomoeae 2 10 ng
P. phaseoli
Negative Control
P. infestans US-23 10 ng

Figure 4.3 A. Amplification curve, B. amplification rate, C. melting temperature, and D. melting curve of *Phytophthora infestans*, *Phytophthora mirabilis*, *Phytophthora ipomoeae*, and *Phytophthora phaseoli* with our LAMP assay. *P. ipomoeae* was amplified very late compared to *P. infestans*. Our LAMP assay detected *P. infestans* at ~8 min and *P. ipomoeae* was amplified very late ~18 min. Our LAMP assay did not detect *P. mirabilis* and *P. phaseoli* (*Phytophthora andina* not included, A and B). Our LAMP assay had the annealing temperature of ~ 86ºC (C and D).

4.4.2 Sensitivity of loop-mediated isothermal amplification (LAMP) assays

The different LAMP assays were compared to determine the lowest amount of DNA material they could detect or limit detection in LAMP reactions on the Genie IIIc (Optigene, UK). Using pure cultures, Hansen's ITS, Hansen's Rg86_2, and our LAMP assays (Fig 4.4) had the lowest detection limits at 0.001 ng/ µL (tp: 17.3, 18.46 and 20.45 min, respectively). The Ammour's LAMP assay was able to detect as low as 0.01 ng/µL (tp 4.7 min) and the Khan's LAMP assay down to 1 ng/µL (tp 22.2 min; Table 4.7).

Using DNA extracted from infected leaves using PEG buffer (peg DNA), the Hansen's ITS assay had the lowest detection limit, up to 5-fold dilution (approximately 0.0005 ng/µL), followed by Ammour's, Hansen's Rgn86_2, and our LAMP assay (3-fold dilution, about 0.05 ng/µL). Khan's assay didn't amplify at all.

Using DNA extracted from infected leaves and purified using a Kingfisher mL (KF DNA), the Hansen's ITS assay had the lowest detection limit at up to a 5-fold dilution (0.01 ng/µL), followed by Ammour's and our LAMP assays (Fig 4.4) at a 3-fold dilution. In contrast, Hansen's Rg86_2 LAMP assay detection was only a 2-fold dilution. Khan's assay didn't amplify at all.

Among Sybr and TaqMan assay, Lees TaqMan assay had the lowest limit of detection (0.001 ng/µL) for pure culture (Table 4.7). Atallah's, Khan's, and Llorente's Sybr assays had limits of detection of 0.01ng/µL to 0.05 ng/µL for pure culture and peg DNA. The Bohm's TaqMan assay had limit of detection from 0.05 ng/µL to 0.001 ng/µL for peg DNA, KF DNA and DNA from pure culture.

Table 4.7 Assay performance of different loop-mediated isothermal amplification (LAMP) and qPCR assays using DNA from a different source and extraction methods.

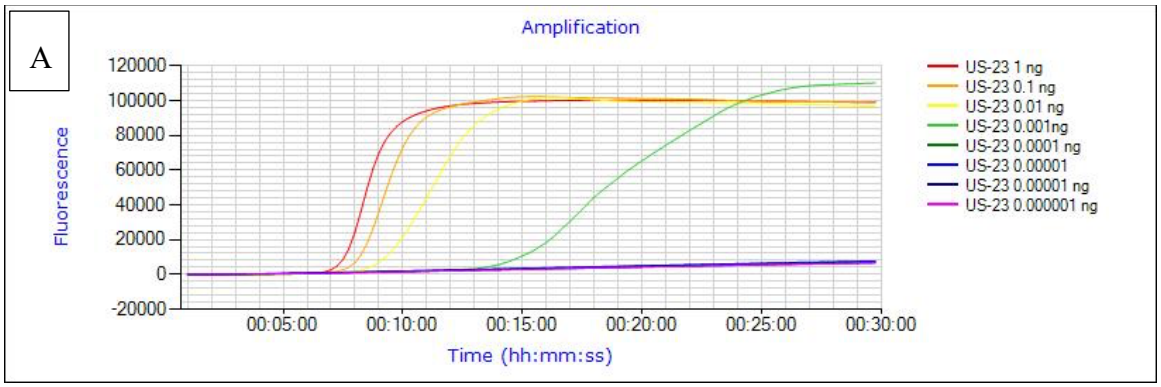
LAMP/qPCR Assays	Time to positive (Tp) /Cycle threshold (Ct) value for lowest limit of detection	RXN efficiency (qPCR assay)	Limit of detection (LOD)	Annealing Temperature (LAMP)
Si Ammour				
Culture – KF[a]	5.73		0.01 ng/µL	87.4
Leaf-PEG[b]	6.16	-	0.05 ng/µL	87.4
Leaf – KF[c]	5.88		0.5 ng/µL	87.4
Hansen (ITS)				
Culture – KF	17.30		0.001 ng/µL	86.7
Leaf-PEG	18.16	-	0.005 ng/µL	86.6
Leaf – KF	19.23		0.005 ng/µL	86.4
Hansen (Rg86_2)				
Culture – KF	18.46		0.001 ng/µL	89.7
Leaf-PEG	9.31	-	0.5 ng/µL	89.6
Leaf – KF	13.58		0.5 ng/µL	89.4
Khan				
Culture – KF	22.2		1 ng/µL	88.1
Leaf-PEG	0	-	-	-
Leaf – KF	0		-	-
Ristaino				
Culture – KF	22.66		0.1 ng/µL	88.2
Leaf-PEG	19.09	-	0.5 ng/µL	88.6
Leaf – KF	17.01		5 ng/µL	88.7
Current study				
Culture - KF	20.45		0.001 ng/µL	86.0
Leaf-PEG	12.30	-	0.05 ng/µL	86.3
Leaf – KF	17.73		0.05 ng/µL	85.6
Atallah (SYBR)				
Culture - KF	45.0	98.37	0.01 ng/µL	-
Leaf-PEG	41.2	-	0.05 ng/µL	
Leaf – KF	43.1	105.95	0.05 ng/µL	
Khan (SYBR)				
Culture - KF	39.4	98.23	0.01 ng/µL	-
Leaf-PEG	37.4	84.41	0.05 ng/µL	
Leaf – KF	30.7	91.91	0.5 ng/µL	
Llorente (SYBR)				
Culture - KF	37.1	89.68	0.01 ng/µL	-
Leaf-PEG	35.9	104.10	0.05 ng/µL	
Leaf – KF	31.1	96.35	0.5 ng/µL	
Böhm (TaqMan)				
Culture - KF	34.3	101.67	0.001 ng/µL	-
Leaf-PEG	29.9	105.93	0.05 ng/µL	
Leaf – KF	28.1	110.17	0.05 ng/µL	
Lees (TaqMan)				
Culture - KF	38.0	100	0.0001 ng/µL	-
Leaf-PEG	37.3	-	0.05 ng/µL	
Leaf – KF	33.6	97.93	0.005 ng/µL	

[a] Culture-KF: genomic DNA extracted from pure culture using robotic Kingfisher mL protocol.

[b]Leaf-PEG: crude DNA extracted from the naturally infected leaf using polyethylene glycol (PEG) buffer.

[c]Leaf-KF: genomic DNA extracted from the naturally infected leaf using robotic Kingfisher mL protocol

Note: qPCR assays were run for 45 min in Bio-Rad CF-connect (Bio-Rad, USA), and LAMP assays were run in Genie II or IIIc (Optigene, UK) for 25 min.


A
Amplification
Fluorescence
120000
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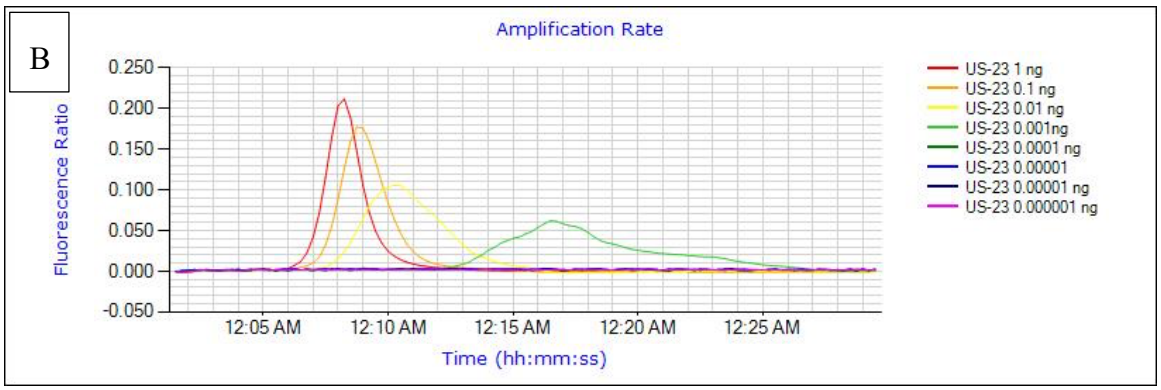
80000
60000
40000
20000
0
-20000
00:05:00
00:10:00
00:15:00
00:20:00
00:25:00
00:30:00
Time (hh:mm:ss)
US-23 1 ng
US-23 0.1 ng
US-23 0.01 ng
US-23 0.001ng
US-23 0.0001 ng
US-23 0.00001
US-23 0.00001 ng
US-23 0.000001 ng


B
Amplification Rate
Fluorescence Ratio
0.250
0.200
0.150
0.100
0.050
0.000
-0.050
12:05 AM
12:10 AM
12:15 AM
12:20 AM
12:25 AM
Time (hh:mm:ss)
US-23 1 ng
US-23 0.1 ng
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US-23 0.001ng
US-23 0.0001 ng
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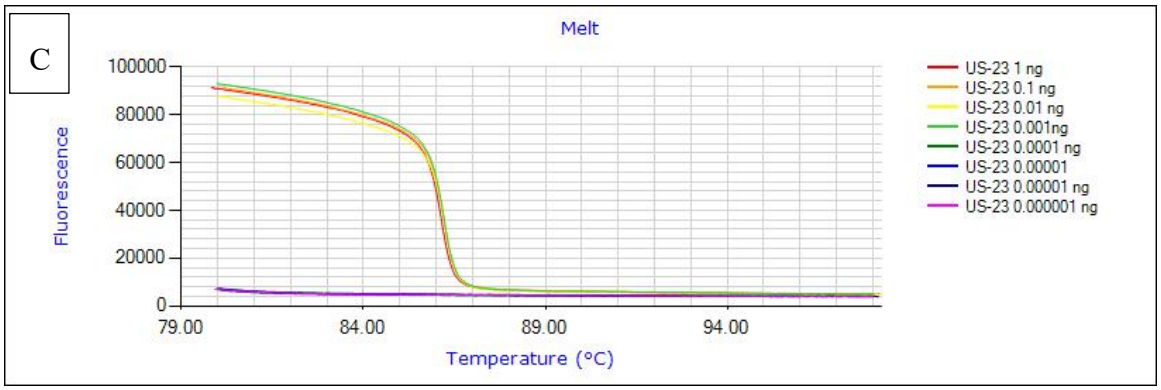
C
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100000
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20000
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79.00
84.00
89.00
94.00
Temperature (°C)
US-23 1 ng
US-23 0.1 ng
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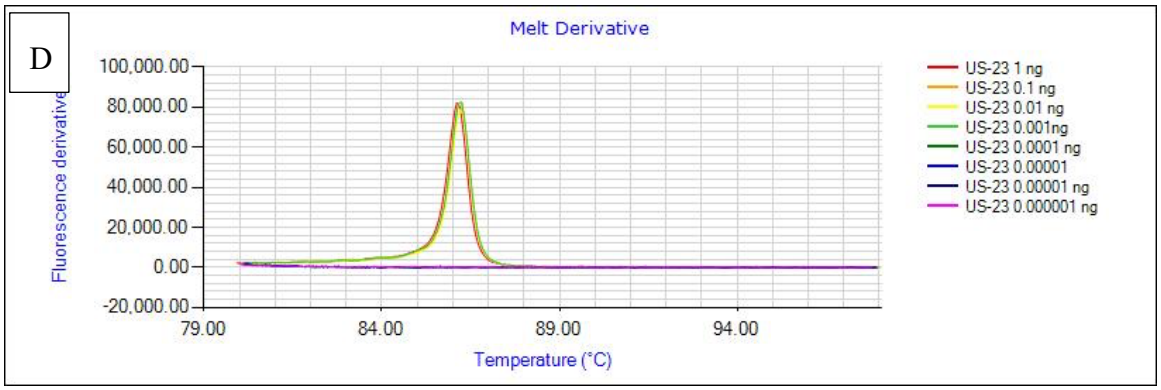
US-23 0.001ng
US-23 0.0001 ng
US-23 0.00001
US-23 0.00001 ng
US-23 0.000001 ng


D
Melt Derivative
Fluorescence derivative
100,000.00
80,000.00
60,000.00
40,000.00
20,000.00
0.00
-20,000.00
79.00
84.00
89.00
94.00
Temperature (°C)
US-23 1 ng
US-23 0.1 ng
US-23 0.01 ng
US-23 0.001ng
US-23 0.0001 ng
US-23 0.00001
US-23 0.00001 ng
US-23 0.000001 ng

4.4d.

Figure 4.4 Limit of detection experiment to determine the lowest limit of DNA concentration that our current LAMP assay could detect consistently and precisely. A. Amplification curve and B. amplification rate showing the detection of different concentration of *Phytophthora infestans* starting at ~8 min (1 ng/μL) and the least at ~ 22 min (0.001 ng/μL), C. melting temperature, and D. melting curve showing the annealing temperature of ~ 86 ºC.

4.4.3 Performance of different master mix

The N001 and N004 isothermal master mix (UK) was compared. N004 Isothermal master mix was faster than N001. The N004 reduced the time to positive (tp) value by 2-3 min. Stem primers were mixed to check whether these primers reduced the tp value. Stem primers did not significantly reduce the tp value (Table 4.8).

Table 4.8 Comparison of loop-mediated isothermal amplification (LAMP assay) using the original master mix N001 and newer master mix N004. DNA from two different extraction methods used in the comparison assay. Stem primers were included in one test and excluded in another.

LAMP assay	MasterMix	Primers	Tp - PEG	SE	Tp - KF	SE
Current study	N001	No Stem	12.15	0.85	13.16	1.85
Current study	N001	Plus Stem	10.38	0.93	10.81	0.35
Current study	N004	No Stem	8.23	0.22	8.31	0.22
Current study	N004	Plus Stem	8.24	0.13	7.73	0.27

4.5 Discussion

The main objective of this study was to develop a *P. infestans* specific and sensitive LAMP assay that could be used in the field or on-site. This study developed a LAMP assay to detect *P. infestans* at the field level coupled with a quick DNA extraction method (alkaline PEG buffer) and carried it out on the Genie II/IIIc platform. Crude DNA can be extracted within 5 min using alkaline PEG buffer that can be used in the LAMP assay right away after diluting the DNA to lower pH (around 7) without further DNA isolation steps (Chomczynski & Rymaszewski, 2006). We designed our LAMP assay using the sequence from the ras-related protein *ypt1* gene, which is species-specific and has less cross-reaction to the closely related species of *P. infestans* in clade 1c. This LAMP assay provides rapid detection of *P. infestans* within 10 min and has the advantage over other time-consuming and labor-intensive methods. The LAMP assay developed in this study was very sensitive. It could detect 1pg of target DNA extracted from pure culture and 3-fold dilution of crude DNA extracted using alkaline PEG buffer from the naturally infected leaf.

Loop-mediated amplification assays have many advantages over traditional PCR-based molecular amplification methods (Khan et al, 2017; Tomlinson et al, 2012). Recently, a few LAMP assays have been developed to detect *P. infestans* at the field level using ITS sequence or ras-related protein *ypt1* gene. However, some cross-react with close relatives of *P. infestans* or take a long time to give positive results. Most published assays developed for *P. infestans* are based on the colorimetric change in reaction (Si Ammour et al, 2017; Hansen et al, 2016; Khan et al, 2017; Ristaino et al, 2019). Some assays need the addition of dye after the completion of the reaction to determine positive or negative results. Opening tubes to add dyes increase the risk of contamination in the field. These assays can be run on the Genie platform. However, the results for sensitivity for each assay might differ. Since we need a rapid, accurate, and species-specific assay, any close relative of *P. infestans* present in the field may contaminate the sample, and a non-specific LAMP assay may cross-react, giving false-positive results. Therefore, a LAMP assay designed for field or on-site testing needs to be species-specific or cross-react with few/ limited pathogens as much as possible. Furthermore, the LAMP reaction should be completed in one step to reduce the risk of contamination as in LAMP assays based on colorimetric change.

The second objective of our study was to compare our assay with other existing LAMP assays to detect *P. infestans*. In this experiment, we compared our LAMP assay with those already published assays using the Genie platform. All the assays were sensitive to *P. infestans*. However, the time to positive was different for different assays. The LAMP assay developed by Si Ammour et al (2017) was most sensitive to *P. infestans*. However, it cross-reacted with all the close relative species of *P. infestans* on clade 1c. Similarly, the assays developed by Hansen et al (2016) were sensitive. However, the LAMP assay designed using ITS sequence cross-reacted with all the close relative species of *P. infestans* on clade 1c. LAMP assay designed by Hansen et al (2016) using the sequence of Rgn86_2 cross-reacted with *P. mirabilis*, *P. ipomoeae,* and *P. andina*; however, *P. ipomoeae* was detected very late in the reaction. The LAMP assays developed by Khan et al (2017) and Ristaino et al. (2019) detected *P. infestans* too late in the reaction. The LAMP assay designed by Ristaino et al. (2019) also cross-reacted with close relatives of *P. infestans* on clade 1c. The LAMP assays developed by Khan et al (2017) did not cross-react with close relatives of *P. infestans*. However, this assay has reduced sensitivity compared to other assays (ran 25 min on Genie IIIc). Khan assay was designed without loop primers, and it has been reported that the LAMP primers without loop primers are less sensitive and take more time to detect the target (Gandelman et al, 2011).

All the assays used in the study were designed from the ITS region except Hansen (Rgn86_2), Khan, and our assay. These ITS-based assays were more sensitive (except for Ristaino et al. 2019). However, they showed reduced specificity in agreement with the original papers. Compared with other LAMP assays, our assay was sensitive and more specific and could detect *P. infestans* within 10 min. The sensitivity of our LAMP assay revealed a consistent 1 pg DNA detection threshold which was comparable to or better than other LAMP and qPCR assays. Our LAMP assay was also compared with the qPCR assays based on Sybr and TaqMan. Among qPCR assays, assays based on the TaqMan probe were more sensitive and had 100 % reaction efficiency than Sybr assays. Our LAMP assay was better than both Sybr and TaqMan probe assays based on specificity and comparable to TaqMan probe assays in terms of sensitivity. Among the qPCR assays, the assay developed by Lees et al (2012) was very sensitive, detecting 0.0001 ng/µL DNA from pure culture, and

our LAMP assay was just 10-fold less sensitive (0.001 ng/µL) than Lees qPCR TaqMan assay.

Our third objective was to determine the sensitivity of our LAMP assay and compare it with the existing LAMP assays. Most LAMP assays designed for the detection of *P. infestans* used DNA extracted using standard methods such as DNeasy plant mini kit, freeze-dried samples, and CTAB methods (Si Ammour et al, 2017; Hansen et al, 2016; Khan et al, 2017; Lees et al, 2019; Ristaino et al, 2019). However, these DNA extraction methods are time-consuming, and some are not applicable for field testing. Ristaino et al (2019) had adopted the high alkaline sodium extraction method developed by Wang et al (1993), which is possible to use in-field testing. In our study, we used the alkaline PEG buffer method to extract DNA, which is fast (it takes less than 5 min to extract DNA) and makes it feasible to extract DNA in any place (Chomczynski & Rymaszewski, 2006). We tested different LAMP assays with crude DNA extracted using PEG buffer from naturally infected leaves. We observed variable sensitivity, which did not agree with the published results. We can speculate that assay sensitivity might differ based on DNA extraction methods and DNA quality (crude or genomic). It has been reported that some of the simplified DNA extraction methods might compromise sensitivity compared to standard DNA extraction kits (Tomlinson et al, 2012). When we tested DNA from the same sample but extracted it using different methods (PEG buffer or CTAB methods). In different LAMP assays, we observed that crude DNA was as sensitive as genomic DNA. Our LAMP assay was 1-fold less sensitive on crude DNA than genomic DNA. However, 3-fold diluted PEG DNA and 4-fold diluted genomic DNA were approximately the same when quantified using TaqMan qPCR assays developed by Böhm et al (1999) or Lees et al (2012). One of the disadvantages of DNA extracted using a PEG reagent is the quality of crude DNA. Crude DNA cannot be maintained for long even in the freezer, so it should be used immediately and should not be stored for an extended period. Previous studies have reported the usefulness of simplified DNA extraction methods in LAMP assays (Ristaino et al, 2019; Tomlinson et al, 2010a; Tomlinson et al, 2012). These simplified DNA extraction methods can be used in low-resource settings, thus overweighing its limitation, especially sensitivity (Tomlinson et al, 2012). A high alkaline PEG reagent is best suited for on-site detection of pathogens because it is fast, effective and less expensive

and doesn't require other instruments such as a heat block/water bath or vortex for DNA extraction.

Our LAMP assay was compared with the new and original isothermal master mix, and the new isothermal master mix decreased the time to positive by approximately 3 min. It has been reported that the stem primers decrease the time to positive and increase sensitivity and specificity (Gandelman et al, 2011). In our study, stem primers did reduce time to positive, however, they did not significantly reduce it.

The best way to control late blight is using integrated pest management strategies. *Phytophthora infestans* is polycyclic, and sporulation occurs in a matter of 2-3 days under conducive environmental conditions. The early, rapid, and accurate detection of this pathogen is the key to successful disease control and reducing losses significantly. The early detection of this pathogen helps to take appropriate disease management action on time. The LAMP assay developed in this study can be applied for on-site detection of *P. infestans*, giving rapid and accurate results within 10 min. The early detection of *P. infestans* can provide growers with ample time to decide on a proper disease management strategy. Then samples can be sent to the lab right away to characterize the genotypes of *P. infestans* further because different genotypes have different sensitivity to commonly used Metalaxyl-M or other fungicides (Dangi et al, 2021; Danies et al, 2013; Hu et al, 2012). This assay and the Genie platform could be integrated with a weather-based disease forecasting system to predict late blight more accurately and reduce fungicide spray based on the presence or absence of disease in the field. In the future, LAMP assays coupled with the Genie platform could be developed to distinguish *P. infestans* genotypes right in the field so that the growers could decide the fungicide they want to use to control late blight. Timely diagnosis of disease will significantly reduce the loss from disease and save fungicide sprays, reducing the risk to health and environmental hazards.

5 EFFECT OF *PYTHIUM ULTIMUM* CONCENTRATION IN THE SOIL, ON TUBERS AT HARVEST AND PRE-STORAGE TEMPERATURE ON DISEASE INCIDENCE AND SEVERITY IN STORAGE

5.1 Summary

The disease severity in tubers infected with *Pythium ultimum* was reported to be higher at storage temperatures above 15°C. A systematic study was conducted to determine the level of disease severity at four pre-storage temperatures (15°C, 20°C, 25°C, and 30°C), stored at two storage temperatures, 8.8°C, and 12.8°C, to emulate the condition in commercial fields at harvest. In addition to this study, tubers were artificially inoculated to determine the maximum disease severity level incubated at those pre-storage temperatures and compare the disease severity and incidence with naturally infected tubers. A third experiment was conducted to determine the disease severity and incidence in the tubers harvested from minimally and highly infested soil. Furthermore, a minimum number of oospores of *Pythium ultimum* required to cause infection was also investigated. Overall, the tubers incubated at 15°C and stored at 8.8°C had minimum disease severity and incidence followed by 20°C. Similarly, disease severity and incidence were lower in tubers incubated at 15°C and stored at 12.8°C compared to tubers incubated at 20°C, 25°C, and 30°C. The trend was similar in both naturally and artificially inoculated tubers. However, disease developed more rapidly in artificially inoculated tubers. The disease severity and incidence were higher in tubers harvested from highly infested soil. The DNA levels of *Pythium* in highly infested soil were higher than in the minimally inoculated field. However, we could not correlate the DNA level in soil and disease severity in tubers harvested from those fields. The minimum number of oospores required to cause infection was as low as one oospore. However, significantly higher severity was observed at ten spores and above. We concluded that the *Pythium* inoculum level in the soil directly impacts disease severity in storage. However, the pre-storage temperatures, wounding at harvest, and storage temperatures had the most impact on disease severity and incidence. These findings are in conjunction with several previous studies.

5.2 Introduction

There is little information about the factors such as pre-storage and storage temperature, and inoculum level in the soil affecting Pythium disease severity in storage. Growers need to know if the pathogen is present in soil or on tubers, inoculum level, epidemiology, and more important effect of pre-storage and storage temperature on disease severity to determine the right disease management strategies that can be used before the onset of disease. Therefore, experiments were conducted to determine if soil inoculum level and pre-storage temperature during harvest on disease incidence and severity of tubers in storage. The broad objective of this project was to develop a better understanding of the Pythium leak enhancing factors such as soil inoculum level, pre-storage, and storage temperature to predict the risk of Pythium leak in storage. The objectives were: a) to determine the effect of high and low *Pythium* inoculum in the soil on disease incidence and severity in the storage and b) to determine the effect of pre-storage temperature on disease incidence and severity in storage, c) to determine the minimum number of *Pythium ultimum* oospores required to cause infection in potato tubers.

5.3 Materials and methods

5.3.1 Culture maintenance and inoculum preparation

A single isolate of *Pythium ultimum* var *ultimum* was used throughout the study. *Pythium ultimum* was maintained, and inoculum was prepared as described in Chapter 2.

5.3.2 Level of inoculum in the soil during the growing season

Two row 1.8 m x 7.6 m plots were established as RCBD in 524 field at Aberdeen Research and Extension Center. Each plot was replicated four times. Soil samples were collected from research plots to determine and track the inoculum concentration of *P. ultimum*. Soil samples (100 g) were collected from research plots (total eight plots) at the row-closure stage (two weeks after inoculation) and harvest (two weeks after vine kill) using a W sampling method (even the plots were small). Soil samples were collected from near the potato plants. A thin slice or a layer of soil ~ 15 cm deep was collected using a hand trowel and kept in Ziplock bags labeled with the information of each plot. In 2018, soil samples

were also collected from the highly and minimally inoculated fields three times in a season. Soil samples were stored at 4°C until use. DNA was extracted from 50 g soil using the method described in Chapter 2 which utilizes a CTAB based lysis buffer followed by purification using paramagnetic particles in conjunction with a Kingfisher mL magnetic particle processer (Woodhall et al, 2012). The amount of *P. ultimum* DNA was quantified using *Pythium* species-specific primers (described in Chapter 2, Cullen et al, 2007), TaqMan probe, and qPCR (Bio-Rad). Standard curve was used to quantify the amount of DNA in the samples.

5.3.3 Effect of pre-storage and storage temperatures on disease severity

Potatoes were planted in two rows, 1.8 m x 7.6 m plots in 524 field at Aberdeen Research and Extension Center. Potato cv Russet Burbank was planted on May 11, 2016, and May 16, 2017 (field 524). Plots were maintained and irrigated as described above. In this study, two different experiments were conducted. In first experiment, tubers were naturally infected from Pythium-infested soil during harvest to emulate the condition in the commercial fields, and in second experiment, tubers were artificially inoculated in the lab. Any tubers bruised during harvest were assumed to be infected with *Pythium*. Tubers were harvested, and medium-size tubers were selected for the study. Thirty-five tubers per replication (4 replication) were kept in mesh bags and immediately incubated at either 15°C, 20°C, 25°C, or 30°C for 48 hours in Peltier chambers (Sable system international, North Las Vegas, NV, USA). The relative humidity was maintained at 90-95% using moist paper towels. After 48 hours of pre-storage treatment, tubers were transferred to plastic crates lined and covered with moist burlap bags. Tubers were stored at two storage temperatures: either at 8.8°C or 12.8°. Burlap bags were kept moist by adding water as needed, and the crates were covered to maintain humidity.

For the artificial inoculation experiment, tubers were bruised by dropping from a three-meter high corrugated metal tower. Tubers were inoculated as described in Chapter 2. After inoculation, tubers were transferred to Peltier chambers (Sable system international, North Las Vegas, NV, USA) set at four different pre-storage temperatures as mentioned above. Tubers were placed in plastic crates lined with moist burlap bags and stored at either

8.8°C or 12.8°C in storage. Humidity of plastic crates were maintained as described above. Disease incidence, severity, and pathogens level were determined as described above.

5.3.4 Effect of *Pythium* inoculum level in the soil on disease severity

An experiment was conducted in two fields (524 and 113) locations at Aberdeen research and extension center to determine the effect of *Pythium* concentration in soil on disease severity in storage. Each field was considered a repetition. Potatoes (cv. Russet Burbank) were planted on May 14, 2018 at two different fields (field 524 and 113). Field 524 has been continuously used for Pythium experiments for more than seven years. Crop rotation was practiced in field 113 every year. Wheat and potato were alternatively rotated in field 113. Plots were maintained with a standard fertilizer rate, weeds were killed/maintained with herbicides, and irrigation was done with an overhead sprinkle system. The number of irrigations was maintained the same in both fields. Plots were inoculated with *P. ultimum* suspension (mycelia and sporangia/oospores) at the hilling stage. Eighteen meter long (two rows,1.8 meters wide) plot was inoculated with high inoculum (10^4 sporangia/oospores per mL). Another 18 meter long (two rows,1.8 meters wide) plot was inoculated with low (1:1000 dilution) inoculum compared to a high inoculum field. These two plots were separated with two-row buffer plots. Tubers were collected arbitrarily from each plot at harvest. The sample size for storage trials was thirty-five tubers per replication and four replications in total. Tubers were kept in mesh bags. Tubers were transferred to plastic crates lined and covered with moist burlap bags. Tubers were stored at two storage temperatures: either at 8.8°C or at 12.8°C (commercially, 8.8°C is used for fresh market potatoes and 12.8°C for processing potatoes). So, in total, there were two sets of tubers in plastic crates from each field (low or highly infested plots from each field) for 8.8°C or 12.8°C storage. Moisture was checked every day, and burlap bags were sprayed with sterile distilled water as needed to maintain high humidity. Disease incidence and severity were recorded at seven-day intervals, and DNA was extracted from the same tubers (~ 30-100g) using a CTAB tissue DNA extraction protocol in a Kingfisher mL. *Pythium* DNA was quantified as described above.

5.3.5 Disease assessment and statistical analysis

Tubers were cut longitudinally regardless of the size, and the disease severity was visually estimated. The percentage of volume taken up by the Pythium leak symptoms was

recorded for disease severity. Later disease severity percentage was averaged for five tubers (from the same replication that week). Disease incidence was determined by counting the number of tubers with Pythium leak symptoms and rated as 0 (absence of symptom) or 1 (presence of symptom), later changed to percentage. DNA data were transformed as log(x+1). The disease severity, incidence, and DNA pg/g data were analyzed using MANOVA and ANOVA on JMP software (version 5.0.1. SAS Institute Inc., Cary, NC, 1989–2021). Estimated means were separated using Tukey HSD ($\alpha = 0.05$). A regression model for disease severity, disease incidence and DNA pg/g of tuber tissue was selected after comparing other models for best fit in Sigmaplot (version 10.0. Systat Software, San Jose, CA). The cubic model was best fitted to some disease severity or incidence data. However, the overall sigmoidal model was best fitted in most disease severity and incidence data. So, the sigmoidal model was chosen for our data analysis. Sigmoidal model represented by $F = a/(1+\exp^{-(x-x_0)/b)})$ where F = percentage of disease severity or incidence or DNA pg/g, x = potato stored duration at different temperatures (weeks), a, b and x_0 are parameters as described in Table 5.6.

5.4 Results

5.4.1 Level of inoculum in the soil

The concentration of *P. ultimum* in the soil was tested two weeks after inoculation and at harvest using species-specific primers and a TaqMan probe. The result showed a decrease in *P. ultimum* concentration by approximately 40% between hilling and harvest (Fig. 5.1). However, this was enough to cause infection in tubers stored at two different temperatures in storage. The DNA amount in the soil was lower in 2017 compared to 2016. However, disease incidence and severity were higher in 2017. The discrepancy in the inoculum level of two years was unknown.

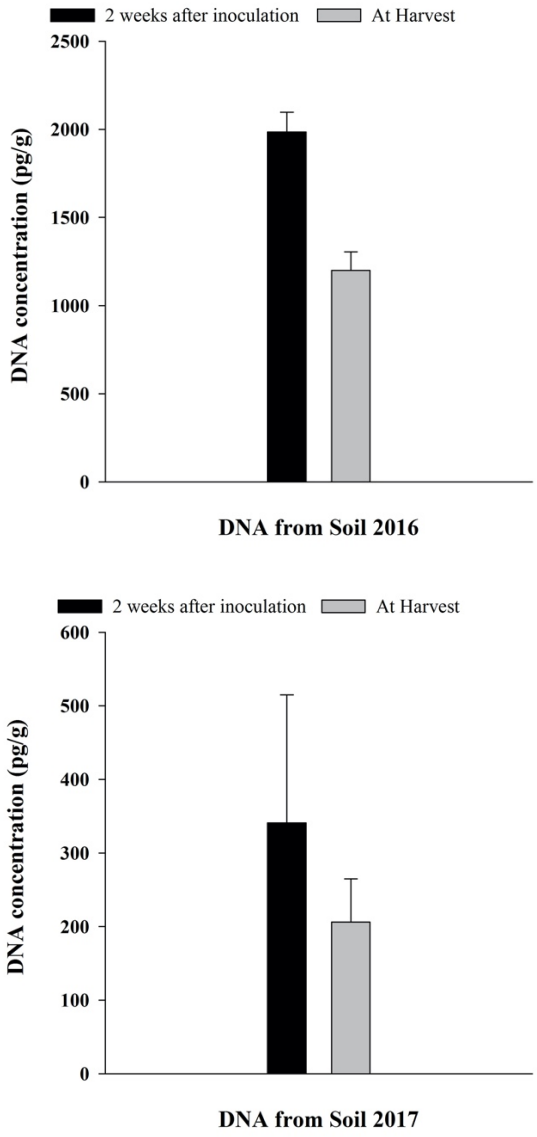


Figure 5.1 DNA from soil samples collected two weeks after *Pythium* inoculum sprayed in the field and at harvest. The DNA concentration in pg/g of soil. In both years (2016 and 2017), *Pythium* DNA level was higher at two weeks after inoculation and lowest at harvest. *Pythium* DNA decreased during the growing season.

In another experiment, the concentration of *P. ultimum* in the soil was tested in high and low inoculum-infested fields three times in a season. The interaction between field and sampling time was significant (Table 5.1). Means of DNA pg/g soil at pre-inoculation in 524 field was different than other sampling time and field. The inoculum level was high after planting, which decreased over time and lowest at harvest. After inoculation and at harvest, the DNA level was not significantly different (Table 5.1). Similarly, the DNA level at planting and after inoculation was not significantly different. However, the inoculum level was significantly different in high and low *Pythium* infested fields (Table 5.1). The 524 field was continuously used for the Pythium trials for at least 8-10 years. The inoculum level was higher in field 524 compared to field 113.

Table 5.1 Least square means of inoculum level in two different fields at three different sampling time. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Field	*Pythium* DNA pg/g of soil	
524	177.5	a
113	80.6	b
Sampling Time		
Pre-inoculation (T1)	205.7	a
After inoculation (T2)	126.9	ab
Pre-harvest (T3)	54.4	b
Field x Sampling Time		
524 Pre-inoculation (T1)	331.4	a
524 After inoculation (T2)	163.5	b
113 After inoculation (T2)	90.3	b
113 Pre-inoculation (T1)	80.1	b
113 Pre-harvest (T3)	71.3	b
524 Pre-harvest (T3)	37.5	b

5.4.2 Disease incidence and severity in tubers incubated at four pre-harvest temperatures

A two-way ANOVA was conducted to compare each pre-storage temperature for two years. The interaction between pre-storage temperatures and week (storage duration) were not significant in both 8.8°C and 12.8°C in 2016 except for disease severity at 12.8°C. However, in 2017 the interaction between pre-storage temperatures and week (storage duration) were significant in both 8.8°C and 12.8°C except for DNA pg/g at 8.8°C storage temperature. The pre-storage temperatures, 15°C, 20°C, and 25°C, were not significantly different in terms of disease severity and incidence between years at 8.8°C storage temperature (data not shown). However, the DNA level differed at 20°C and 25°C between two years. Disease incidence and severity of tubers incubated at all pre-storage temperatures (except 20°C) and stored at 12.8°C storage temperature was significant between years. Disease severity and incidence of tubers incubated at pre-storage temperatures and stored at 12.8°C were significant and less significant at 8.8°C storage temperatures, so data were not combined.

Disease severity between pre-storage temperatures in 2016 at 8.8°C storage temperature was not different (Table 5.2). However, disease incidence and disease progression per week was significant. The disease level between pre-storage temperatures was not significantly different. The disease severity between tubers incubated at pre-storage temperatures and stored at 12.8°C in 2016 was significantly different. However, disease incidence was not different (Table 5.2). The DNA level of *Pythium* in tubers was not significant at 12.8°C in 2016 among pre-storage temperatures; however, DNA differed in disease progression over time (per week). The disease severity, incidence, and DNA pg/g between pre-storage temperatures in 2017 at 8.8°C and 12.8°C storage temperatures were significantly different from each other except for DNA pg/g in the interaction between week (storage duration) and pre-storage temperatures at 8.8°C (Table 5.3).

The tubers displayed significantly visible symptoms after week 3 in both years (Table 5.4). The tubers started showing disease symptoms that increased exponentially after three weeks in storage in both years and temperatures. Our experiment took three weeks for naturally infected tubers to develop the significantly higher disease. However, they were not

statistically significant between week 0 to week 4 (Table 5.4). Disease severity, incidence, and DNA pg/g increased over time and were significantly highest at weeks 5 and 6 in both storage temperatures.

The tubers incubated at pre-storage temperatures, 25°C and 30°C, had higher infection compared to the tubers incubated at 15°C (Table 5.5). The storage temperature of 8.8°C had lower disease severity than 12.8°C in both years (Table 5.5). Disease severity was not significantly different in 2016 in tubers stored at 8.8°C. However, disease incidence was significantly different in tubers incubated at 20°C and 25°C. The disease severity in tubers incubated at 20°C and 25°C (pre-storage temperatures) and stored at 12.8°C was significantly different from disease severity in tubers incubated 15°C (pre-storage temperature). However, disease incidence was the same for all pre-storage temperatures. The DNA level was not significantly different in all tubers stored at both temperatures. Overall, in 2017 we observed higher disease severity and incidence compared to 2016. The DNA level was also higher in 2017. The tubers incubated at pre-storage temperatures of 25°C and 30°C had higher disease severity, incidence, and DNA pg/g compared to the tubers incubated at 15°C and 20°C except in tubers incubated at 25°C and stored at 8.8°C in terms of DNA. The tubers incubated at a pre-storage temperature of 15°C had the lowest disease severity, incidence, and DNA level in both storage temperatures (Table 5.5).

A sigmoidal regression model best fitted our data compared to other models. This model best explained the disease progression over time (weeks). The equation of the fitted sigmoidal model was detailed in materials and methods, and estimated parameters are shown in table 5.6 (data only shown for 2017). Since, 2016 data did not best fit any model, the data are not shown. Figure 5.2 and 5.3 represents the graphs used to fit sigmoidal regression, and each graph has equation and R^2 values. The pre-storage temperature of 30°C had consistent disease severity, incidence, and DNA, so the sigmoidal regression model was best fitted with a coefficient of determination (R^2) above 87%, except in DNA. Since the DNA was extracted from five tubers of a replication, none of the regression model best fit (R^2 value too low). However, sigmoidal regression model was chosen for DNA because this model was best among other models.

Table 5.2 Two-way analysis of variance for effects of pre-storage temperatures, week, and resulting interaction for disease severity, incidence, and DNA pg/g in 2016. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
8.8°C				
Disease Severity				
Pre-storage Temperature	3	82.09821	2.2259	0.0911
Week	6	331.60714	4.4953	0.0005
Pre-storage Temperature x Week	18	221.46429	1.0007	0.4675
Disease Incidence				
Pre-storage Temperature	3	1300	4.2326	0.0078
Week	6	5950	9.686	<.0001
Pre-storage Temperature x Week	18	2650	1.438	0.1356
DNA pg/g				
Pre-storage Temperature	3	15.657211	0.9549	0.418
Week	6	47.981855	1.4631	0.2008
Pre-storage Temperature x Week	18	80.354452	0.8168	0.6758
12.8°C				
Disease Severity				
Pre-storage Temperature	3	130.92393	6.3869	0.0006
Week	6	755.29429	18.4228	<.0001
Pre-storage Temperature x Week	18	251.66857	2.0462	0.0153
Disease Incidence				
Pre-storage Temperature	3	114.2857	0.6667	0.5748
Week	6	5885.7143	17.1667	<.0001
Pre-storage Temperature x Week	18	1085.7143	1.0556	0.4103
DNA pg/g				
Pre-storage Temperature	3	2.605036	0.9668	0.4124
Week	6	28.132046	5.2205	0.0001
Pre-storage Temperature x Week	18	13.141039	0.8129	0.6803

Table 5.3 Two-way analysis of variance for effects of pre-storage temperatures, week, and resulting interaction for disease severity, incidence, and DNA pg/g in 2017. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
8.8°C				
Disease Severity		2825.6071	15.5421	<.0001
Pre-storage Temperature	3	3019.7143	8.3049	<.0001
Week	6	2024.1429	1.8556	0.0314
Pre-storage Temperature x Week	18	2825.6071	15.5421	<.0001
Disease Incidence				
Pre-storage Temperature	3	16185.714	18.128	<.0001
Week	6	11921.429	6.676	<.0001
Pre-storage Temperature x Week	18	9964.286	1.86	0.0309
DNA pg/g				
Pre-storage Temperature	3	856099328	4.0588	0.0096
Week	6	1548454001	3.6706	0.0028
Pre-storage Temperature x Week	18	1629626814	1.2877	0.2171
12.8°C				
Disease Severity				
Pre-storage Temperature	3	12187.312	62.7663	<.0001
Week	6	5794.982	14.9225	<.0001
Pre-storage Temperature x Week	18	6420.375	5.511	<.0001
Disease Incidence				
Pre-storage Temperature	3	42467.857	36.1429	<.0001
Week	6	18385.714	7.8237	<.0001
Pre-storage Temperature x Week	18	16157.143	2.2918	0.0059
DNA pg/g				
Pre-storage Temperature	3	2311829743	38.8457	<.0001
Week	6	2420142093	20.3328	<.0001
Pre-storage Temperature x Week	18	2099929014	5.8808	<.0001

Table 5.4 Disease progression in potato tubers incubated at 15°C, 20°C, 25°C and 30°C pre-storage temperatures for 48 hours and stored in 8.8°C and 12.8°C for six weeks. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Storage duration (weeks)	Disease Severity %		Disease Incidence %		DNA pg/g	
	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C
Year 2016						
0	0.0 c	0.0 d	0.0 b	0.0 c	0.0 a	0.0 b
1	0.2 bc	0.8 cd	2.5 b	2.5 c	0.3 a	0.5 ab
2	0.0 c	0.1 d	0.0 b	1.3 c	0.2 a	0.5 ab
3	0.3 bc	0.1 d	5.0 b	1.3 c	1.2 a	1.4 a
4	2.3 abc	3.5 bc	8.8 ab	11.3 b	0.9 a	1.2 a
5	4.2 a	5.6 ab	17.5 a	13.8 ab	0.9 a	0.9 ab
6	3.8 ab	6.5 a	18.8 a	20.0 a	2.1 a	1.4 a
Year 2017						
0	0.0 c	0.0 c	0.0 d	0.0 c	4.9 b	4.9 b
1	0.9 c	1.9 c	5.0 cd	1.9 c	22.7 b	6.1 b
2	2.8 c	7.0 bc	8.8 bcd	7.0 bc	240.2 b	912.9 b
3	4.8 bc	7.3 bc	22.5 abc	7.3 bc	1123.0 ab	4198.4 b
4	6.5 bc	13.1 ab	23.8 ab	13.1 ab	3337.0 ab	4480.2 b
5	11.9 ab	17.6 a	25.0 ab	17.6 a	9354.6 a	11358.5 a
6	14.9 a	20.6 a	27.5 a	20.6 a	8250.0 ab	11643.8 a

Table 5.5 Overall disease severity over time (weeks) in potato tubers stored at 8.8°C and 12.8°C, 48 hours after incubation at four different pre-storage temperatures (15°C, 20°C, 25°C, and 30°C). Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Pre-storage temperature (°C)	Disease Severity (%)		Disease Incidence (%)		DNA pg/g	
	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C
Year 2016						
15	0.3 a	0.6 b	2.9 b	5.7 a	0.7 a	0.6 a
20	2.0 a	3.4 a	11.4 a	8.6 a	0.5 a	0.8 a
25	2.6 a	3.1 a	10.0 a	7.1 a	1.4 a	1.0 a
30	1.3 a	2.3 ab	5.7 ab	7.1 a	0.6 a	1.0 a
Year 2017						
15	0.2 c	0.0 c	0.7 c	0.0 c	59.1 b	13.4 c
20	4.0 bc	4.6 bc	12.9 b	10.7 bc	1442.0 b	2681.6 bc
25	5.9 b	6.8 b	16.4 b	20.7 b	3926.0 ab	3774.5 b
30	13.9 a	27.2 a	34.3 a	52.1 a	7334.3 a	12161.8 a

Table 5.6 Estimated parameters of the sigmoid equation for the relationship between the disease severity of tubers and pre-storage temperatures at both storage temperatures in 2017 with disease severity (*F*) and time in the week (x) together with a coefficient of determination R^2 and *p*-value.

Storage temperature	Pre-storage temperature	Regression terms				
		a	b	X_0	R^2	*P*-value
8.8°C	15	0.7	0.0	4.4	0.41	0.3403
8.8°C	20	21.8	1.4	6.0	0.85	0.0106
8.8°C	25	29.8	1.5	5.8	0.98	0.0050
8.8°C	30	41.4	1.2	4.3	0.99	0.0001
12.8°C	15	0.0	1.0	0.0	1.00	(NAN)
12.8°C	20	37.3	1.7	7.1	0.72	0.0350
12.8°C	25	412.8	2.5	13.9	0.89	0.0051
12.8°C	30	57.1	1.0	3.2	0.99	0.0001

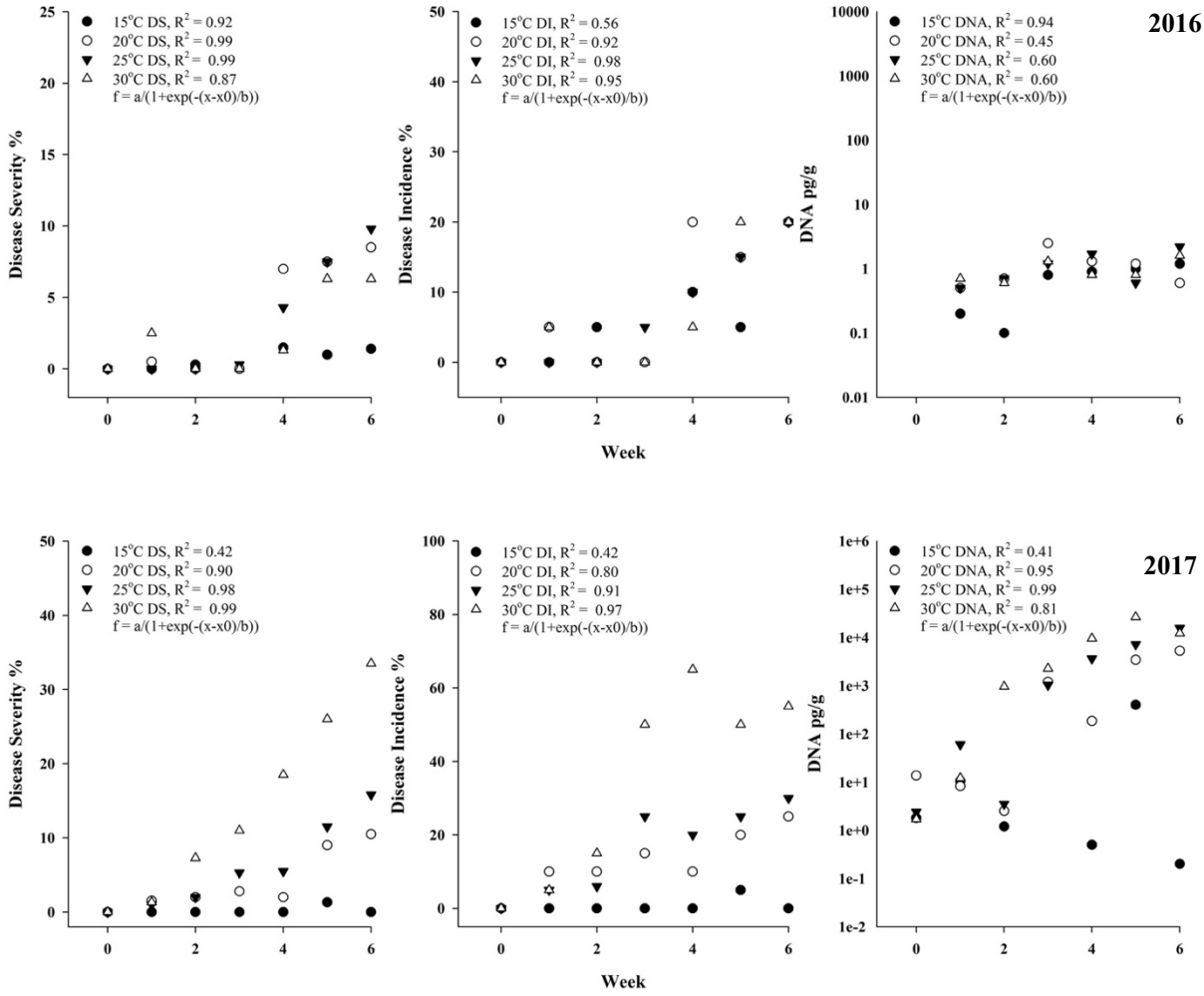


Figure 5.2 Regression analysis of naturally infected tubers, incubated at 15°C, 20°C, 25°C and 30°C pre-storage temperatures for 48 h and then stored at 8.8°C for six weeks (Top graph for 2016 and bottom graph for 2017). The sigmoidal regression equation and R^2 value of each curve are given in the legend. Disease severity, disease incidence, and *Pythium* DNA pg/g of tuber tissue (in most cases) increased over 6 weeks period. Disease severity, disease incidence, and *Pythium* DNA pg/g of tuber tissue were higher in 2017 compared to 2016.

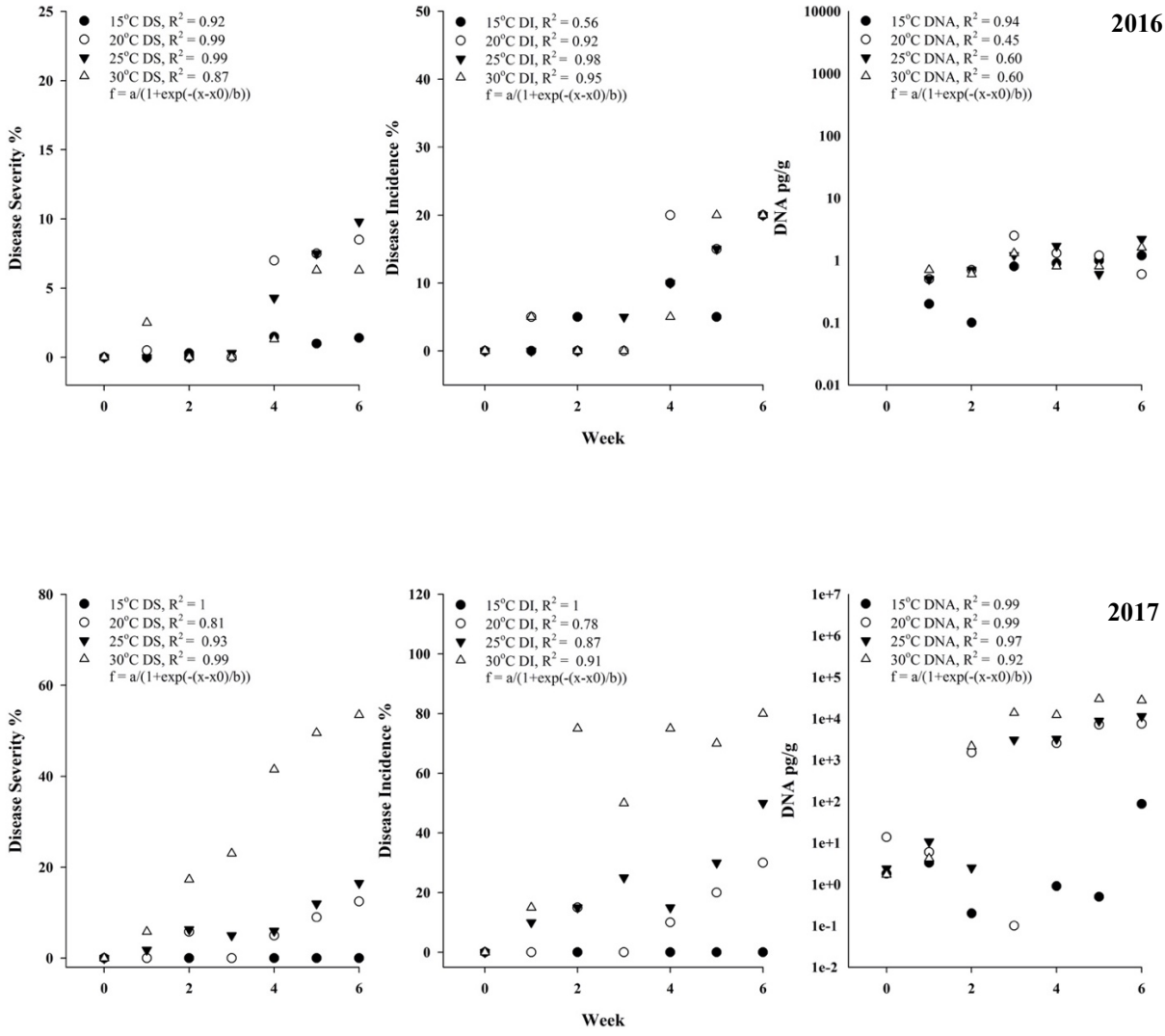


Figure 5.3 Regression analysis of naturally infected tubers, incubated at 15°C, 20°C, 25°C and 30°C pre-storage temperatures for 48 h and then stored at 12.8°C for six weeks (Top graph for 2016 and bottom graph for 2017). The sigmoidal regression equation and R^2 value of each curve are given in the legend. Disease severity, disease incidence, and *Pythium* DNA pg/g of tuber tissue (in most cases) increased over 6 weeks period. Disease severity, disease incidence, and *Pythium* DNA pg/g of tuber tissue were higher in 2017 compared to 2016.

5.4.3 Disease incidence and severity in artificially inoculated tubers

A two-way ANOVA was conducted to compare each pre-storage temperature in two experiments. Tubers inoculated and incubated at pre-storage temperatures 15°C (for disease incidence), 20°C (for DNA), and 25°C (disease severity and DNA) for 48 hours and then stored at 8.8°C (storage temperature) were not significantly different between years (data not shown). The tubers inoculated and incubated at pre-storage temperatures 15°C, 20°C and 25°C for 48 hours and then stored at 12.8°C for six weeks were not significantly different between years in terms of disease severity or incidence or DNA pg/g of tissue. Tubers inoculated and incubated at pre-storage temperatures 15°C, 20°C, 25°C, and 30°C for 48 hours and stored at 8.8°C and 12.8°C for six weeks were mixed significant/non-significant in terms of disease severity and incidence. Therefore, data were not combined.

The disease severity between 15°C, 20°C, 25°C, and 30°C pre-storage temperatures in experiment 1 (Exp1) and experiment 2 (Exp2) at both storage temperatures was significant (effect data not shown, Table 5.7 and 5.8). The disease incidence and disease progression per week were significant (Table 5.7). The DNA levels between pre-storage temperatures were also significantly different from the pre-storage temperatures.

The tubers showed significant visible symptoms after week 2 in run 1 and week 1 in experiment 2 (Table 5.7). The tubers started showing disease symptoms that increased exponentially after three weeks in experiment 1 and 2 weeks in experiment 2 in both years and storage temperatures. The latent period of *Pythium ultimum* to develop disease was shortened in artificial inoculation. Our experiment took as little as 1 week for artificially infected tubers to develop significantly higher symptoms. The disease severity, incidence, and DNA pg/g increased over time in both storage temperatures and had a significantly highest level at weeks 4 to 6. The disease severity, incidence, and DNA pg/g were lowest in 0-2 weeks in experiment 1 and 0 and 1 week in experiment 2.

The tubers incubated at pre-storage temperatures of 25°C and 30°C had higher infection compared to the tubers incubated at 15°C and 20°C (Table 5.8). The tubers incubated at pre-storage temperatures and stored at 12.8°C had developed the highest disease severity compared to tubers stored at 8.8°C. The disease severity and incidence of pre-storage temperatures 15°C and 20°C were not significantly different in tubers stored at 8.8°C and

12.8°C in experiment 1 with the exception of disease incidence at 12.8°C storage temperature. However, disease incidence was significantly different in experiment 2 except at 8.8°C storage temperature. The disease severity was significantly higher in tubers incubated at 30°C in both storage temperatures. The DNA level was significantly different in all tubers incubated at 30°C and stored at 8.8°C temperatures. Since the tubers were inoculated by soaking for 16 h, the DNA level was not significantly different in most of the pre-storage temperatures in both storage temperatures (Table 5.8)

The sigmoidal regression model was also fitted in artificial inoculation experiment data. The equation of the fitted sigmoidal model and R^2 values are shown in each graph (Fig 5.4 and 5.5). The pre-storage temperature of 30°C had consistent disease severity and incidence, and the best fit model was sigmoidal with a coefficient of determination (R^2) above 90% except in DNA pg/g. The DNA pg/g had low R^2 for all the tested models.

Table 5.7 Weekly disease progression in terms of disease severity, incidence, and DNA pg/g in potato tubers artificially inoculated, incubated at 15°C, 20°C, 25°C, and 30°C for 48 h, and then re-stored at either 8.8°C or 12.8°C for six weeks. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Storage duration (weeks)	Disease Severity (%)		Disease Incidence (%)		DNA pg/g	
	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C
Experiment 1						
0	0.0 c	0.0 b	0.0 b	0.0 c	0.0 b	0.0 b
1	0.0 c	0.0 b	0.0 b	0.0 c	0.0 b	0.0 b
2	2.9 c	9.0 b	38.8 a	45.0 b	75630.0 a	56311.4 a
3	9.7 b	29.9 a	42.5 a	56.3 b	67750.0 a	121076.5 a
4	13.6 b	27.6 a	53.8 a	46.3 b	35393.6 a	42771.8 a
5	20.0 a	38.3 a	53.8 a	72.5 a	53774.6 a	154433.7 a
6	NA	NA	NA	NA	NA	NA
Experiment 2						
0	0.0 c	0.0 d	0 b	0.0 c	0.0 c	0.0 b
1	4.4 bc	6.0 d	51.3 a	66.3 ab	87543.2 ab	71287.5 b
2	17.9 ab	24.0 c	58.8 a	53.8 b	352214.6 a	533197.6 a
3	25.5 a	29.2 bc	70.0 a	72.5 a	4484.1 b	23088.6 b
4	28.6 a	32.4 bc	63.8 a	70.0 ab	5556.7 b	5339.2 b
5	28.8 a	38.2 b	57.5 a	57.5 ab	3772.6 b	372.5 b
6	31.3 a	54.3 a	50.0 a	58.8 ab	37109.3 b	50816.6 b

*NA = did not run in that experiment.

Table 5.8 Disease severity, incidence, and DNA pg/g in potato tubers artificially inoculated, incubated at 15°C, 20°C, 25°C, and 30°C for 48 h, and then re-stored at either 8.8°C or 12.8°C for six weeks.

Pre-storage temperature (°C)	Disease Severity (%)		Disease Incidence (%)		DNA pg/g	
	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C
Experiment 1						
15	1.3 c	1.1 c	11.7 b	2.5 c	130.1 b	56.0 b
20	1.8 c	7.1 c	12.5 b	34.2 b	8874.8 b	12727.0 b
25	11.4 b	25.0 b	48.3 a	50.8 a	24683.4 b	116957.0 a
30	16.3 a	36.7 a	53.3 a	59.2 a	121343.8 a	115116.0 b
Experiment 2						
15	3.7 c	3.8 d	22.1 b	15.7 c	66.1 b	105593.2 a
20	13.2 b	17.1 c	35.7 b	36.4 b	22487.7 ab	78335.6 a
25	17.5 b	31.1 b	65.0 a	78.6 a	104315.1 ab	148923.8 a
30	43.5 a	53.2 a	77.9 a	85.7 a	153520.0 a	58062.9 a

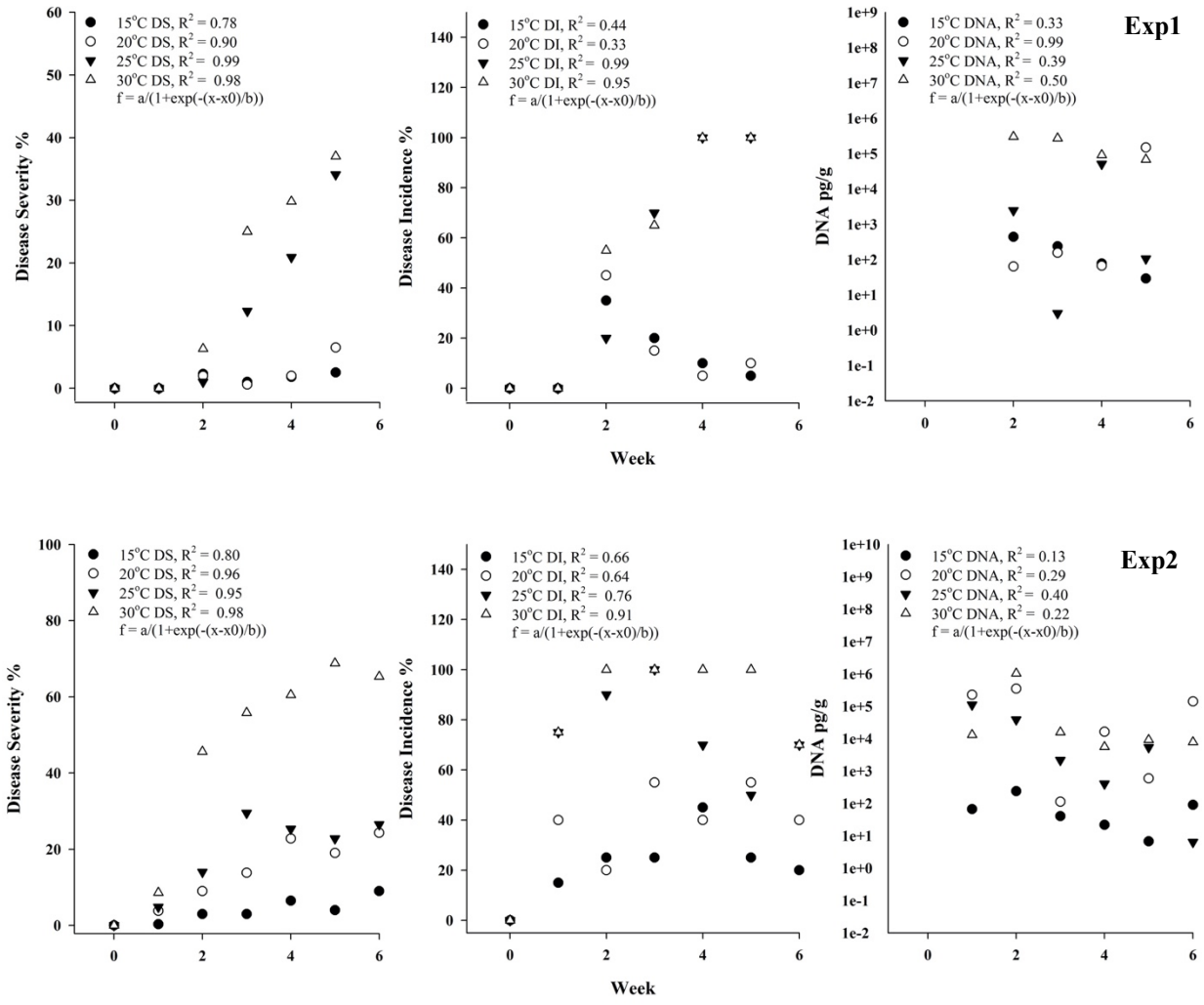


Figure 5.4 Regression analysis of tubers artificially inoculated, incubated at 15°C, 20°C, 25°C, and 30°C for 48 h, then re-stored at 8.8°C for six weeks. The sigmoidal regression equation and R^2 value of each curve are given in the legend. Disease severity and disease incidence increased over 6 weeks period (except *Pythium* DNA pg/g potato tuber). Disease severity and disease incidence were higher in experiment 2 compared to experiment 1.

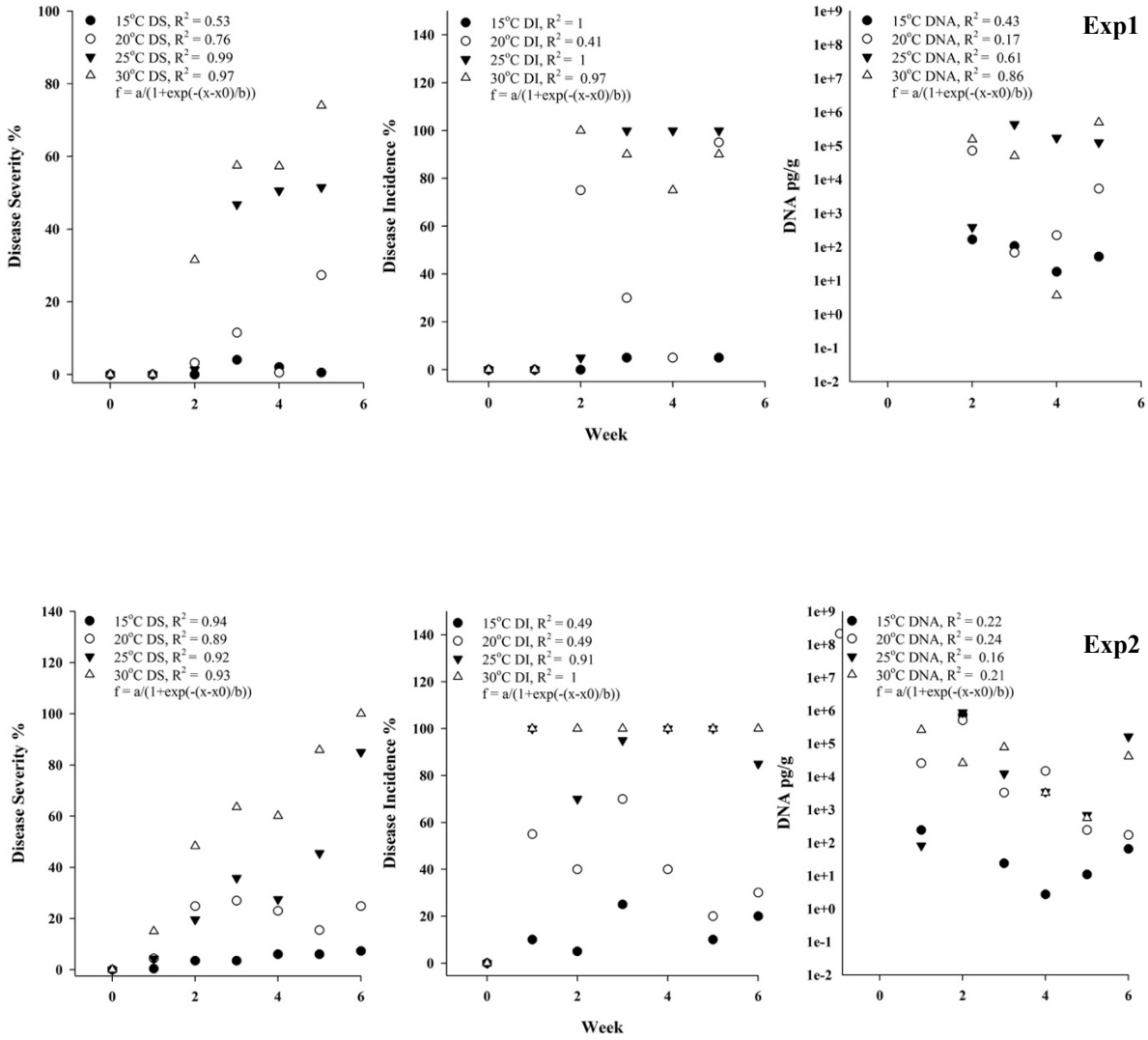


Figure 5.5 Regression analysis of tubers artificially inoculated, incubated at 15°C, 20°C, 25°C, and 30°C for 48 h, then re-stored at 12.8°C for six weeks. The sigmoidal regression equation and R^2 value of each curve are given in the legend. Disease severity and disease incidence increased over 6 weeks period (except *Pythium* DNA pg/g potato tuber). Disease severity was higher in experiment 2 compared to experiment 1. Disease incidence had similar trend in both experiments.

5.4.4 Disease incidence and severity in tubers harvested from highly and minimally infested soil.

A two-way ANOVA was conducted to compare each storage temperature in two fields. The disease severity, incidence, and DNA of tubers harvested from highly infested soil were not significant. Similarly, the disease severity, incidence, and DNA of tubers harvested from minimally infested fields were not significantly different. So, the data from two fields were combined. The highly infested fields were combined as one set of data and minimally infested fields were combined as another set of data.

The disease severity, incidence, and DNA for tubers from soil were not significantly different at 8.8°C storage temperature. However, the disease progression per week was significant at 12.8°C storage temperature (Table 5.9). The disease progressed over six weeks and reached a maximum at weeks 3 and 4. The disease severity, incidence, and DNA for tubers from soil were significantly different at 12.8°C storage temperature and was highest in tubers harvested from a highly infested field. The tubers stored at 12.8°C storage temperature had higher infection compared to the tubers incubated at 8.8°C (Table 5.9). Overall, tubers were infected and showed symptoms at 8.8°C and 12.8°C storage temperatures. However, the disease did not develop as high as those incubated at four different temperatures and stored at the same temperature as in this experiment.

The disease severity, incidence, and DNA for tubers surface vs. disease severity were significantly different at both 8.8°C and 12.8°C storage temperatures, except for the tubers harvested from high and low inoculum and stored at 8.8°C. The disease severity, incidence, and DNA were highest at week 5. Similarly, the tubers harvested from highly infested soil were significantly different from those harvested from minimally infested soil (Table 5.10).

The sigmoidal regression model was also fitted for high and low inoculum level experiment data. The equation of the fitted sigmoidal model and R^2 values are shown in each graph (Fig 5.7). The storage temperature of 12.8°C had consistent disease severity and incidence in both soils vs. disease severity and inoculum on tuber surface vs. disease severity, so the sigmoidal regression model was best fitted with a coefficient of determination (R^2) above 89%.

Table 5.9 Disease severity, incidence, and DNA pg/g of tubers harvested from minimally and highly infested soil with *Pythium* mycelia/oospores stored at 8.8°C and 12.8°C for six weeks. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Storage duration (weeks)	Disease Severity (%)		Disease Incidence (%)		DNA pg/g	
	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C
0	0.0 a	0.0 b	0.0 a	0.0 b	0.0 a	0.0 b
1	1.9 a	0.0 b	4.1 a	0.0 b	4.1 a	0.0 b
2	0.9 a	2.7 ab	7.5 a	7.5 ab	7.5 a	7.5 ab
3	1.7 a	5.4 a	7.5 a	12.5 a	7.5 a	12.5 a
4	3.6 a	7.1 a	6.3 a	10.0 a	6.3 a	10.0 a
5	1.4 a	5.4 a	7.5 a	6.3 ab	7.5 a	6.3 ab
Pythium Concentration						
High	2.2 a	6.4 a	6.4 a	10.0 a	6.4 a	10.0 a
Low	1.0 a	0.4 b	4.6 a	2.1 b	4.6 a	2.1 b

Table 5.10 Disease severity, incidence, and DNA pg/g of tubers harvested from minimally and highly infested soil with *Pythium* mycelia/oospores on tuber surface, stored at 8.8°C and 12.8°C for six weeks. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Storage duration (weeks)	Disease Severity (%)		Disease Incidence (%)		DNA pg/g	
	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C	Storage Temp 8.8°C	Storage Temp 12.8°C
0	0.0 b	0.0 b	0.0 b	0.0 b	6.5 d	6.5 b
1	0.6 ab	1.9 b	1.3 ab	6.3 ab	67.8 cd	89.8 b
2	2.6 ab	4.3 ab	2.5 ab	10.0 ab	94.1 cd	114.9 b
3	0.6 ab	3.5 ab	6.3 ab	6.3 ab	126.8 bc	119.5 b
4	0.9 ab	5.3 ab	7.5 ab	7.5 ab	169.2 ab	147.9 b
5	3.4 a	8.0 a	10.0 a	13.8 a	9610.7 a	44857.7 a
Pythium Concentration						
High	1.9 a	7.1 a	6.7 a	11.7 a	2397.1 a	11128.2 a
Low	0.8 a	0.5 b	2.5 b	2.9 b	961.2 b	3983.9 b

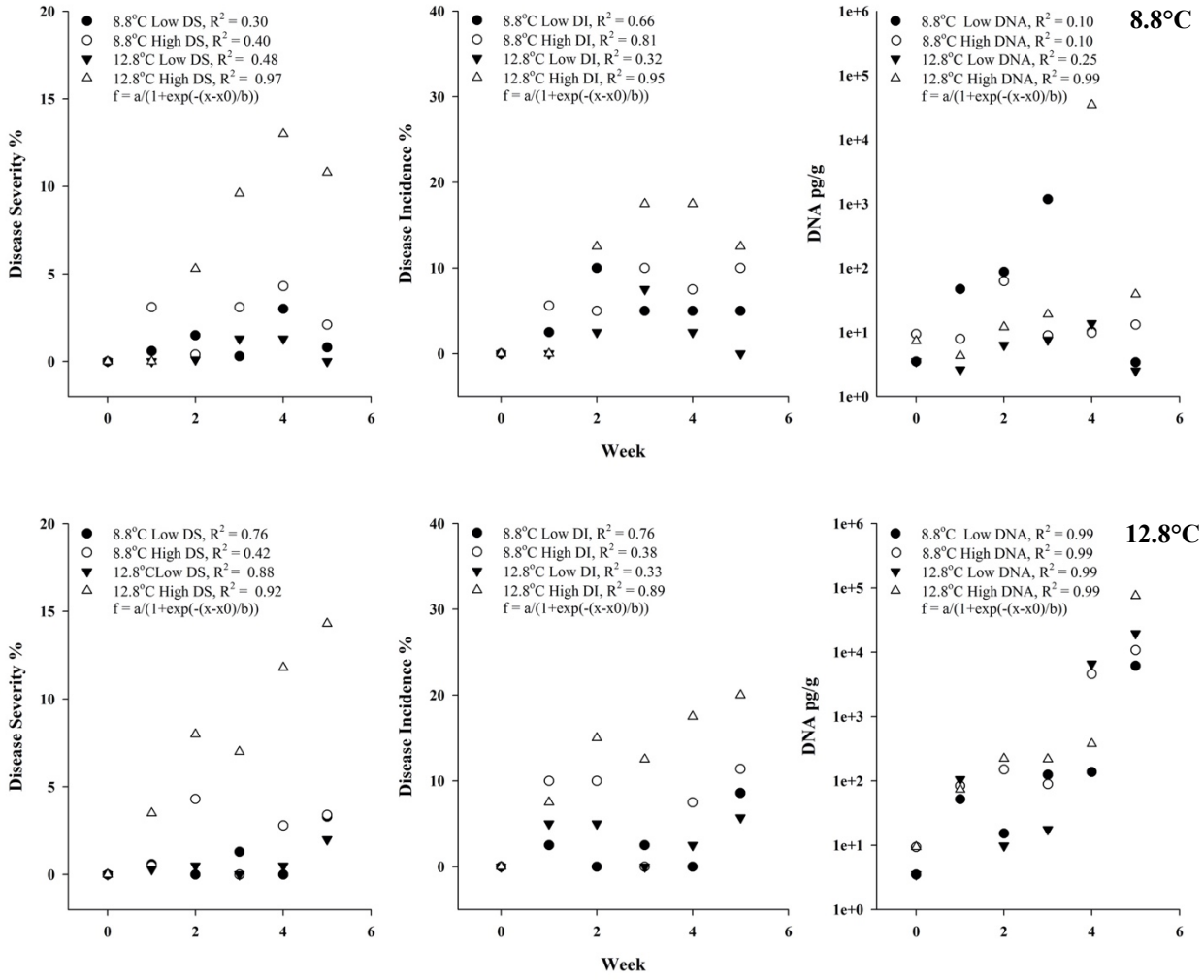


Figure 5.6 Regression analysis of tubers harvested from minimally and highly infested soil with *Pythium* mycelia/oospores stored at 8.8°C and 12.8°C for six weeks. The sigmoidal regression equation and R2 value of each curve are given in the legend. The top row is the graph for disease severity, incidence, and DNA vs. *Pythium* inoculum in the soil. The bottom is the graph for disease severity, incidence, and DNA vs. *Pythium* inoculum on the tuber surface (naturally infected).

5.4.5 Disease incidence and severity in tubers inoculated with different numbers of oospores

The number of oospores required to cause infection in tubers and the DNA from those oospores was determined in this experiment. Disease severity and incidence increased with an increase in the number of oospores and over 31 days after inoculation (Table 5.11). A single oospore was enough to cause infection. However, we observed inconsistent performance of a single spore. Consistent disease severity and incidence were observed in tubers inoculated with 10 and 100 oospores.

We quantified the DNA of each concentration of oospores, 1, 10, and 100, and found that one oospore yielded a similar Ct value when compared to 10 oospores. The expected Ct value should be in the range of three Ct differences between 10-fold diluted samples/oospores in this case. However, we did not observe three Ct differences per 10-fold diluted samples/or per set of oospores. During DNA extraction, any residue left in DNA extraction might impact the results.

Table 5.11 Disease severity and incidence and DNA pg/g of tubers harvested from minimally and highly infested soil with *Pythium* mycelia/oospores on tuber surface, stored at 8.8°C and 12.8°C for six weeks

Number of Oospores	DNA (Ct)	Disease Severity (DAI*)				Disease Incidence (DAI)			
		10	17	24	31	10	17	24	31
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	33.0	0.6	24.8	19.8	31.5	10.0	30.0	30.0	40.0
10	32.84	0.9	38.8	53.3	64.0	10.0	40.0	50.0	45.0
100	31.34	28.3	44.3	44.5	54.0	45.0	50.0	45.0	65.0

*DAI: Days after inoculation. Tubers were inoculated by injecting inoculum.

5.5 Discussion

There are some previous studies on the epidemiological factors of Pythium leak of potato such as pre-storage temperature, storage temperature, and their effect on disease development potential in storage. Such information is helpful for disease management strategies. In this study, we present the effect of a range of pre-storage temperatures that enhance the potential of disease development in storage. We were also interested to see if there was a correlation between highly infested soil and disease severity/incidence in storage. The minimum number of *Pythium* infective propagules needed to cause disease was also determined along with these factors. Our study's approach was to emulate the conditions at harvest in commercial potato fields and storage.

Previous studies were conducted in the lab or in storage by artificially infecting the potato tubers, bruising, wounding, or injecting the inoculum in the tubers (Lui & Kushalppa, 2003; Salas et al, 2003; Taylor et al, 2008). These studies demonstrated that the maximum disease severity was observed at 20°C with 48 h wetness period or at 21°C (Hollingshead et al, 2020; Lui & Kushalppa, 2003). The temperature range for severe infection of potato tubers by *P. ultimum* has been reported to be between 20-30°C (Salas & Secor, 2001). Lui & Kushalppa (2003) developed a model to predict potato tuber infection by *Pythium* in the lab and storage, which is very useful for the growers. However, all these studies were limited to lab and artificial inoculation methods. Artificial inoculation with the wounding method may overestimate the disease severity or incidence more than those naturally infected during harvest on the harvester. Furthermore, inoculating tubers in the lab provides high-density inoculum pressure, which might not be accurate in the field, on the harvester or during the handling process. *Pythium* needs a wound or open lenticels to cause infection in potato tubers (Lifshitz & Hancock, 1984). The pathogen cannot directly penetrate the potato periderm. So, in this study, we have used both naturally and artificially infected tubers to give a close estimate of disease severity in the storage in both cases. Furthermore, we have also done correlation studies between the disease severity from highly infested soil and minimally infested soil.

In the current study, we evaluated the disease severity of tubers that were naturally infected on the harvester or artificially wounded in a corrugated metal bruiser by dropping

tubers from a three-meter height of a metal bruiser and tubers from a highly and minimally infested field. We observed high disease severity and incidence in tubers which were incubated at 20°C, 25°C, and 30°C for 48 h and stored at 12.8°C. Disease severity and incidence were significantly limited in the tubers incubated at 15°C and stored at 8.8°C. Overall, disease severity and incidence were low at 8.8°C compared to 12.8°C. Our results agreed with the findings of other studies that Pythium leak severity decreases and is low below 10°C (Burton, 1948; Salas & Secor, 2001). In artificially inoculated tubers, we observed severe Pythium leak symptoms within 2-3 weeks, and by the end of week 6, those tubers with Pythium leak were infected with secondary bacteria. The disease severity and incidence might differ in different cultivars. Some studies have been conducted on disease severity and disease screening on different varieties of potatoes (Hollingshead et al, 2020; Salas & Secor, 2003; Thompson et al, 2007). Most of the cultivars were susceptible to *Pythium*, except the susceptibility was different among different cultivars.

Our study observed variation in disease severity within the same incubation temperature. This can be attributed to the multiple infection point during the harvest. A similar observation was reported by Salaas et al (2001) when screening cultivar resistance. Salaas et al (2001) had reported that some cultivars had more severity than others due to multiple entry points for the pathogen. However, Salaas et al (2001) did not observe differences in disease incidence. We can also speculate that there could be variation in skin set (periderm development) in the tubers grown in the different seasons because the growing conditions might not be the same every year. Skin set directly impacts bruising, skinning, or wounding during harvest (Lulai, 2007). The wet soil towards the end of the season also affects the skin set process. The saturated soil also enhances the swelling of lenticels. The potato tubers with swollen lenticels and susceptible to bruising and skinning increases the potential of *Pythium* propagules contamination or pathogen transmission during harvest. The wet soil during harvest increases the chances of tuber infection (Lifshitz & Hancock, 1984). We had observed the potato growing condition was different in 2016 and 2017. In 2017, we had wet soil towards the end of the season and during harvest. This can be the reason for high disease severity and incidence in that year.

Our results showed that artificially inoculated tubers had a shorter latent period than naturally infected tubers. Artificially inoculated tubers had a higher amount of inoculum in

the inoculum suspension, fresh deep bruises, and a more extended inoculation period than the tubers naturally infected in the field and at harvest which is significantly low in time and inoculum. This most likely has a direct impact on differences in the latent period. Other studies by Schippers (1977) had reported that the respiration of potato tubers decreases with the fall of temperature in long-term storage. The pathogens remain latent at low temperatures and cause diseases when they find conducive conditions (Johnson & Cummings, 2009). The low temperature of storage decreases tubers' respiration rate, which reduces the infection activity and increases the latent period of *P. ultimum* (Lui & Kushalappa, 2003). Lower holding temperatures were reported to decrease the rate of disease progression in the storage (Olsen et al, 2006). So, the pre-storage temperatures directly impacted disease severity in the storage.

We also found low inoculum density in the soil in 2017 compared to 2016. However, disease severity and incidence were higher in the storage in 2017. The weather has a direct impact on the survivability of *Pythium* propagules. Adverse weather, such as below 9°C or above 27°C, has a direct effect, and these temperatures tend to decrease the viable propagules of *Pythium ultimum* in the soil (Lifshitz & Hancock (1984). The *Pythium* inoculum had been reduced in 2017. However, the environment was conducive to infection or contamination at harvest because of wet soil. We found that a single oospore can cause infection and speculate that a low inoculum level can still produce high disease severity and incidence in storage if the environment is conducive for the pathogen during harvest because *Pythium ultimum* can infect and cause disease even with a single oospore.

In conclusion, the finding of this study could be useful to develop a disease prediction model because we have covered all four aspects of the epidemiology of Pythium leak in potatoes. To give an accurate and precise forecast, we need to consider all the factors that can significantly impact the disease outcome. When we avoid or miss the weather condition at harvest, pathogen profile in the soil, inoculum density, or limit of pathogen propagules to cause disease, we might overestimate or underestimate the disease in storage, which can be misleading to growers. Growers might end up using unsuitable management strategies to control the disease in the field or the storage. This study's main takeaway findings are to control the Pythium leak appropriately, we need to control or suppress the inoculum level in the field, proper irrigation towards the end of the season, proper skin set with the help of vine

kill, harvesting potatoes during cool mornings and storing them at an appropriate temperature below 15°C. The time after harvest for the potatoes being kept in storage should be decreased as much as possible.

6 EVALUATION OF COMMERCIALLY AVAILABLE FOLIAR FUNGICIDES APPLIED IN THE SEASON TO EFFECTIVELY REDUCE PYTHIUM LEAK AND PINK ROT DISEASE IN STORAGE

6.1 Summary

Pythium ultimum and *Phytophthora erythroseptica* cause severe storage rot disease in potatoes. Since there are very limited chemical controls of these pathogens, we evaluated seven commercially available in-season foliar systemic fungicides at various rates and application timings to see if those fungicides are effective in reducing Pythium Leak and pink rot disease in storage. In addition, the pathogen level in soil was tested three times at different growing stages of potato to ensure pathogen inoculum was maintained until the potatoes were harvested. Stem and tubers of potatoes from different fungicide treated plots were also tested to ensure the pathogen was in the potato. After the potatoes were harvested, they were kept in storage at 12.8C for two to three months before challenge inoculation. Challenge inoculation of potato tubers from different fungicide plots determined that both rates of Resist 57 consistently reduced the Pythium and pink rot disease severity and incidence. However, these two treatments were not significantly different from non-treated control in some years in the Pythium trial. The other fungicide Orondis Gold had reduced some Pythium leak disease. However, it was not significantly different than the non-treated control. In the pink rot trial, fungicides such as both rates of Resist 57, Omega 500F, Orondis Gold, and Ranman significantly reduced pink rot severity and were significantly different than the non-treated control. The DNA level in the soil was high right after inoculation, and it was sufficiently maintained until harvest. The DNA from stem and tuber was confirmed with species-specific primers of *P. ultimum* and *P. erythroseptica*. The positive result of these pathogens in qPCR confirmed that these pathogens were in the potato. In our study, in-season foliar application of fungicides did not increase the yield nor have any phytotoxicity.

6.2 Introduction

Potatoes can get injured or bruised during harvest and can be easily contaminated with any diseased potatoes or pathogen inoculum in the soil. The diseased potatoes or the inoculum in the soil can contaminate the wounded potatoes in the harvester's belt and these contaminated wounded tubers can become a huge problem in the storage contaminating the whole lot of potatoes. The worst scenario in the storage can be the whole lot of potatoes can be melted away due to primary and secondary pathogen/bacterial infection. Integrated management strategies have been used to control the Pythium leak and pink rot in storage. The cultural practices to manage *Pythium ultimum* and *Phytophthora erythroseptica* involve field selection with well-draining soil, crop rotation with non-host crops, limiting irrigation towards the end of the growing season before harvest, giving tubers enough time to set skin after vine kill, and harvesting tubers during cool, dry conditions (Lambert and Salas 2001; Powelson and Rowe 2008). However, cultural practices are insufficient to control the Pythium leak and pink rot in storage. In-season foliar fungicides are a critical component in the integrated disease management system. There are very few effective fungicides for protecting tubers directly in the storage. So, the priority is to use in-season foliar fungicides and cultural practices during the growing season, harvest, and handling operations. There are commercial fungicides available for in-season foliar application in the field. The commercially available fungicides need to be evaluated during growing seasons to check their potential to control or prevent the Pythium leak and pink rot in the storage. The objectives of this study are i) to evaluate the commercially available foliar fungicides at various application timing and rates to see if those fungicides are effective to reduce Pythium Leak and pink rot disease in storage, ii) to monitor the status of *P. ultimum* and *P. erythroseptica* inoculum level in the soil, stem, and tubers of potato.

6.3 Materials and Methods

6.3.1 Cultivar, inoculum, and fungicides

Each year, Russet Burbank was planted in the second week of May (May 11, 2016, May 14, 2017 and May 14, 2018). A single isolate of *Pythium ultimum* var *ultimum* and *Phytophthora erythroseptica* was used throughout the study. *Pythium ultimum* and *P.*

erythroseptica were maintained, and inoculum was prepared as described in Chapter 2. The isolates of *Pythium ultimum* var *ultimum* and *Phytophthora erythroseptica* were sensitive to mefenoxam.

The fungicides used in this study are listed in Table 6.1.

6.3.2 Field experiments and inoculation

Field experiments were set up to evaluate the effect of foliar fungicides on Pythium leak and pink rot of potato. All experiments were conducted in either field 524 or field 113 at the Aberdeen Research and Extension Center of the University of Idaho from 2016 to 2018. Field 524 had no crop rotation for 7-8 years. Potato had been planted continuously for at least 8 years, and the field was used for Pythium and pink rot trials. However, field 113 had crop rotation with wheat and potato every year. Randomized complete block design (RCBD) was used in Pythium leak and pink rot experiments. The experiments were established as a RCBD with eight treatments (seven foliar fungicides and one non-treated control) and four replications. Each plot was two-row 7.6 meters by 1.8 meters, separated by a 1.5-meter buffer between treatment plots. At least another two buffer rows separated the treatment plot rows. The pink rot trial at field 524 was 6 meters long. Potatoes were planted at 30-cm within row spacing in all experiments. The buffer rows were planted with Russet Norkotah.

Plots were maintained with a standard fertilizer rate, weeds were killed/maintained with herbicides, and irrigation was done with an overhead sprinkle system. The number of irrigations was kept the same in both fields. The fungicide spray was started when the tuber bulking reached 2 cm diameter, and the second spray was after 14 days of the first spray. The fungicides were sprayed twice, or three times based on the treatment. A boom sprayer was used to spray the fungicides.

The experimental plots were inoculated with *P. ultimum* or *Phytophthora erythroseptica* suspension (mycelia and sporangia/oospores) at the row-closure stage, as described in Chapter 2.

Table 6.1 Products evaluated in the study of in-season foliar spray for the control of Pythium leak and Rink rot of potato in storage, including Fungicide Resistance Action Committee (FRAC) code, active ingredient, formulation, rate, and manufacturer

Product name	Active ingredient (FRAC[a] Code)	Formulation[b]	Rate[c]	Application code	Manufacturer
Resist 57 (standard)	Phosphorous acid (33)	6.78 lb/gal (0.812 Kg/L) SC	10pt/a (11.7 L/ha)	ABC	Actagro, LLC
Ranman	Cyazofamid (Group 21)	3.33 lb/gal (0.4 Kg/L) SC	6.1 fl oz/a (0.45 L/ha)	AB	FMC Corporation
Omega 500F	Fluazinam (Group 29)	4.17 lb/gal (0.5 Kg/L) SC	8.0 fl oz/a (0.59 L/ha)	ABC	Syngenta Crop Protection Inc.
Headsup	Extract of *Chenopodium quinoa* saponins	49.65 % W/W WG	3.34 oz wt/a (0.23 Kg/ha)	AB	Plant Protectants Inc.
Resist 57	Phosphorous acid (33)	6.78 lb/gal (0.812 Kg/L) SC	10 pt/a (11.7 L/ha)	AB	Actagro, LLC
Ridomil Gold Bravo	Mefenoxam, Chlorothalonil (Group 4, M5)	3.67 Lb/Gal (0.44 Kg/L) SC	40 fl oz/a (2.9 L/ha)	ABC	Syngenta Crop Protection Inc.
Orondis Gold	Oxathiapiprolin and Mefenoxam (Group 4, 49)	1.67 Lb/Gal (0.2 Kg/L) SC	14 fl oz/a (1.02L/ha)	ABC	Syngenta Crop Protection Inc.

[a] FRAC = Fungicide Resistance Action Committee; FRAC code- number and letters used to distinguish fungicide groups according to their cross-resistance behavior.
(FRAC Code List, 2015. Fungicides are sorted by mode of action (including FRAC Code numbering.)
[b] Formulation= products added to the active ingredient to change its physical characteristic and allow compatibility with the machinery SC = Suspension Concentrate, and WG = Water dispersible granule.
[c] Rate applied as foliar spray per acre (or ha).
A = At nickel size tubers (=full bloom), B =14 days after A, C=14 days after B.

6.3.3 Evaluation of the residual effect of foliar fungicides to control Pythium leak and pink rot in storage

The harvested tubers were stored at 12.8°C until use. Tubers were two to three months old after harvest and used for challenge inoculation experiments. One hundred medium size disease-free tubers per treatment were selected. The tubers were bruised and inoculated, as described in Chapter 2. Each treatment had four replications with twenty-five tubers in each replication. Twenty-five tubers were kept in a mesh bag and inoculated. After storing them at 18°C and high humidity for 30 days, the tubers were evaluated for disease incidence and severity.

6.3.4 Inoculum test in stem, tuber, and soil during the cropping season

The inoculum in the stem was tested towards the end of the season before the vine kill. Five stem samples were taken from each plot. Tuber samples were collected when the potatoes were 2 cm diameter (at bloom and first spray). DNA extraction and qPCR were done as described in Chapter 2. Soil samples (100 g) were collected from each plot (total of 32 plots) after planting before inoculation, row-closure stage (2 weeks after inoculation), and harvest (2 weeks after vine kill) using the W sampling method (even the plots were small). In 2016, soil inoculum for *P. ultimum* was tested twice before inoculation and at harvest. However, soil inoculum was tested three times (pre-inoculation, after inoculation, and at harvest) in 2017 and 2018. Soil samples were stored at 4°C until use. DNA was extracted from 50 g soil using a previously described method which utilizes a CTAB based lysis buffer followed by purification using paramagnetic particles in conjunction with a Kingfisher mL magnetic particle processer (Woodhall et al., 2012). Details of the soil DNA extraction method are described in Chapter 2. The amount of *P. ultimum* and *P. erythroseptica* DNA was quantified using species-specific primers (Cullen et al., 2007), TaqMan probe, and qPCR (Bio-Rad).

6.3.5 Disease assessment and data analysis

The tubers from the challenge inoculation study were longitudinally cut into halves from the point of infection. The disease severity and incidence were visually estimated. The volume covered by the Pythium leak or pink rot symptoms was recorded for disease severity in percentage. Later disease severity percentage was averaged for twenty-five tubers (from

the same replication). Disease incidence was determined by counting the number of tubers with Pythium leak or pink rot symptoms and rated as 0 or 1, later changed to percentage. The disease assessment in *P. ultimum* infected tubers was done right after the tubers were cut open. However, the cut tubers were left for 20 minutes after the tubers were cut open in *P. erythroseptica* infected tubers. After 20 minutes, the *P. erythroseptica* infected tubers developed color characteristics of pink rot disease, and disease severity was estimated based on the volume of symptoms.

DNA data were transformed into a log(x+1) to change any zero or small decimal value to a positive number. The disease severity, incidence, DNA pg/g from the soil, stem, tubers, and yield data were tested for normality on XLSTAT (version 24.1.2, Addinsoft Inc. New York, NY, USA). The data that passed the normality test were subjected to two-way ANOVA, and multiple comparisons test Tukey HSD on JMP software (version 5.0.1. SAS Institute Inc., Cary, NC, 1989–2021). Data that were not significantly different between years were combined following Levene's test on XLSTAT (version 24.1.2, Addinsoft Inc. New York, NY, USA). The disease severity, incidence, DNA pg/g, and yield of different treatment data were compared using Tukey HSD (alpha = 0.05). The data that failed normality test were also run for two-way ANOVA and double-checked using the non-parametric approach to the two-way ANOVA, the Kruskal-Wallis test.

6.4 Results

6.4.1 Evaluation of the residual effect of foliar fungicides to control Pythium leak in the storage

The Pythium disease severity and incidence data were significantly different between years (Table 6.2). So, the data were not combined, and further analyses were done separately. The challenge-inoculated tubers showed various degree of disease severity between treatments ranging from 28.4% to 58.0% in 2016, 6.8% to 17.3% in 2017, 14.1% to 30.0% in 2018 (field 524) and 8.5% to 19.8% in 2018 (field 113; Table 6.2). However, the means of treatments were not different from each other in terms of disease severity except in 2018 (field 113). Resist 57 and Resist 57 standards had significantly lower disease severity and incidence every year compared to non-treated control in both 524 and 113 fields (Table 6.3).

However, disease severity and incidence were not statistically significant in 2016, 2017, and 2018 in tubers collected from Resist 57 and Resist 57 standard (field 524). The only difference between Resist 57 and Resist 57 standard was the number of sprays in a season. Resist 57 was sprayed twice and Resist 57 standard was sprayed three times. However, Resist 57 and Resist 57 standards were not significantly different (Table 6.3), and in 2016, 2017, and 2018 (field 524), Resist 57 standards had lower disease severity and incidence. The disease severity of tubers treated with Omega 500F was different than the control in 2018 (field 113. However, disease incidence was not significantly different. Although Orondis Gold and Ranman did not differ from the non-treated control, they had lower disease severity and incidence. Headsup and Ridomil Gold Bravo did not effectively control Pythium severity and incidence.

Table 6.2 Analysis of variance for the main effects of disease severity and incidence of challenge inoculated potato tubers with *Pythium ultimum*. Potato tubers harvested from eight different fungicides treated plots and stored at 12.8°C were used. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
Disease Severity				
Treatment	7	2018.844	2.0635	0.0549
Year	3	21578.126	51.4627	<.0001
Treatment*Year	21	2226.691	0.7586	0.761
Year 2016				
Treatment	7	2626.2822	0.9959	0.4579
Year 2017				
Treatment	7	275.695	1.5119	0.2107
Year 2018 (field 524)				
Treatment	7	963.4622	0.962	0.4801
Year 2018 (field 113)				
Treatment	7	380.095	4.104	0.0043
Disease Incidence				
Treatment	7	2147.5	1.6333	0.1352
Year	3	31828.5	56.4836	<.0001
Treatment*Year	21	1759.5	0.4461	0.9814
Year 2016				
Treatment	3	15.657211	0.9549	0.418
Year 2017				
Treatment	7	504	0.2983	0.9477
Year 2018 (field 524)				
Treatment	7	715.5	0.2715	0.9591
Year 2018 (field 113)				
Treatment	7	1983.5	6.9965	0.0001

Table 6.3 Effect of in-season foliar spray of seven different fungicides on disease severity and incidence on challenge inoculated potato tubers. Potato tubers were challenge inoculated with *Pythium ultimum* and stored at 18°C for 30 days before disease assessment. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Treatments	Years			
	2016	2017	2018 (524)	2018 (113)
Disease Severity %				
Heads up	44.6 a	11.8 a	16.8 a	13.5 ab
Omega 500F	39.5 a	14.4 a	14.7 a	10.8 b
Orondis Gold	58.0 a	10.3 a	13.0 a	11.9 ab
Ranman	42.1 a	13.0 a	30.0 a	14.3 ab
Resist 57 Std	28.4 a	6.8 a	14.1 a	10.1 b
Resist57	41.1 a	11.3 a	16.2 a	8.5 b
Ridomil Gold Bravo	40.9 a	10.1 a	23.0 a	16.5 ab
Non-treated	57.2 a	17.3 a	22.7 a	19.8 a
Disease Incidence %				
Heads up	94 a	48 a	83 a	88 a
Omega 500F	88 a	56 a	82 a	79 ab
Orondis Gold	88 a	51 a	72 a	75 abc
Ranman	93 a	47 a	81 a	79 ab
Resist 57 Std	79 a	44 a	75 a	63 c
Resist57	88 a	43 a	83 a	66 bc
Ridomil Gold Bravo	94 a	45 a	83 a	77 abc
Non-treated	92 a	46 a	88 a	84 a

6.4.2 Evaluation of the residual effect of foliar fungicides to control pink rot in storage

The same treatments as in the Pythium experiment were used in the pink rot experiment. Statistical analysis showed the homogeneity of variance in fields 524 and 113 ($p \leq 0.1712$; Table 6.4 and 6.5). However, there were significant differences between disease incidence results from fields 524 and 113 ($p \leq 0.001$; Table 6.4 and 6.5). The disease severity data from fields 524 and 113 were combined after Levene's test. All the treatments except Headsup and Ridomil Gold Bravo were significantly different in disease severity than non-treated control (Table 6.6). Omega 500F, Orondis Gold, Ranman, Resist 57 standard, Resist 57 had significantly low disease severity and were not significantly different. None of the treatments were significantly different from each other in terms of disease incidence. Although Resist 57, Resist 57 standard, Orondis Gold, and Ranman were not significantly different compared to non-treated control, these fungicides had worked quite well to control the pink rot disease.

Table 6.4 Analysis of variance for the main effects of disease severity and incidence of challenge inoculated potato tubers with *Phytophthora erythroseptica*. Potato tubers harvested from eight different fungicide treated plots and stored at 12.8°C were used. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
Disease Severity				
Treatment	7	676.7411	4.6822	0.0005
Field	1	39.8477	1.9299	0.1712
Treatment*Year	7	68.4536	0.4736	0.8489
Year 2018 (field 524)				
Treatment	7	330.7997	1.4608	0.2264
Year 2018 (field 113)				
Treatment	7	388.9844	6.1673	0.0003
Disease Incidence				
Treatment	7	1975.0000	1.2375	0.3014
Field	1	6561.0000	28.7763	<.0001
Treatment*Field	7	1807.0000	1.1322	0.3592
Year 2018 (field 524)				
Treatment	7	2855.3455	1.1212	0.3812
Year 2018 (field 113)				
Treatment	7	872.4364	1.4731	0.2221

Table 6.5 Disease severity and incidence in potato tubers from two different fields, challenge-inoculated with *Phytophthora erythroseptica* and stored at 18°C for 30 days. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Year/Field	Disease Severity/Incidence (%)	
Disease Severity		
Year 2018, field 113	11.5	a
Year 2018, field 524	9.9	a
Disease Incidence		
Year 2018, field 113	72.8	a
Year 2018, field 524	52.5	b

Table 6.6 Effect of in-season foliar spray of seven different fungicides on disease severity and incidence. Potato tubers were challenge inoculated with *Phytophthora erythroseptica* and stored at 18°C for 30 days before disease assessment. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Treatments	Disease Severity/Incidence (%)	
Disease Severity %	(524 and 113 fields combined)	
Heads up	11.2 ab	
Omega 500F	7.9 b	
Orondis Gold	8.2 b	
Ranman	10.6 b	
Resist 57 Std	9.7 b	
Resist57	7.7 b	
Ridomil Gold Bravo	12.0 ab	
Non-treated	18.3 a	
Disease Incidence %	2018 (524)	2018 (113)
Heads up	60.0 a	78.0 a
Omega 500F	34.0 a	79.0 a
Orondis Gold	43.0 a	66.0 a
Ranman	55.0 a	71.0 a
Resist 57 Std	60.0 a	65.0 a
Resist57	48.0 a	68.0 a
Ridomil Gold Bravo	57.0 a	77.0 a
Non-treated	62.4 a	74.4 a

6.4.3 Inoculum test in stem and tuber from Pythium leak field trials

All the data for DNA were transformed to log (x+1) for data analysis. The data were analyzed for the normality test. The data that passed the normality test were subjected to two-way ANOVA. Statistical analysis showed a significant difference between years and fields, so the data from each year and field were analyzed separately (Table 6.7 and Table 6.9). Stem DNA from eight different treatments was significantly different in 2016 (Table 6.8). Stem DNA from eight treatments was not significantly different in 2017 and 2018 (field 524 and 113; Table 6.8). Similarly, tuber DNA from eight treatments was not significantly different in 2016 and 2017 (Table 6.8). In 2016, stem from the plots treated with Ranman had less DNA than non-treated control and was significantly different. In 2018, tubers from Resist 57 treated plots (field 524) and Orondis Gold (field 113) had the least DNA compared to other treatments (Table 6.8). DNA from tuber from Resist 57 treated plots (field 524) and Orondis Gold (field 113) were significantly different from the non-treated control (Table 6.8). Although statistically not significant, tubers from plots treated with Ridomil Gold Bravo treated plots had less DNA. DNA from the stem was higher in 2018 (field 113), and DNA from the tuber in 2016 was the highest compared to other years.

Table 6.7 Analysis of variance of the main effects of *Pythium ultimum* DNA (pg/g of tissue) in stem and tuber from different fungicide treatment plots, combined and each year. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
Stem DNA pg/g of tissue				
Combined for all years				
Treatment	7	5.2763	0.8888	0.2629
Year	3	83.9930	33.0145	<.0001
Treatment*Year	21	22.1950	1.2463	0.4676
Year 2016				
Treatment	7	1.6000	3.4943	0.01
Year 2017				
Treatment	7	0.7561	1.0740	0.4097
Year 2018 (field 524)				
Treatment	7	14.9757	1.4328	0.2384
Year 2018 (field 113)				
Treatment	7	7.4392	0.7763	0.4059
Tuber DNA pg/g of tissue				
Combined for all years				
Treatment	7	4.00723	1.4372	0.1994
Year	3	64.1377	53.6743	<.0001
Treatment*Year	21	6.51265	0.7786	0.7382
Year 2016				
Treatment	7	2.89091	0.4115	0.8857
Year 2017				
Treatment	7	0.3039	0.8521	0.5566
Year 2018 (field 524)				
Treatment	7	4.1027	2.7638	0.0295
Year 2018 (field 113)				
Treatment	7	3.3390	1.4378	0.2366

Table 6.8 *Pythium ultimum* DNA (pg/g of tissue) in stem and tuber from the inoculated fields, stem collected before vine kill and tubers at two cm diameter stage, in 2016 (field 524), 2017 (field 524), and 2018 (field 524 and 113). Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Treatments	Years							
	2016		2017		2018 (524)		2018 (113)	
Stem DNA								
Heads up	39.1	ab	40.0	a	642.6	a	6695.2	a
Omega 500F	66.5	ab	47.1	a	1047.2	a	4977.2	a
Orondis Gold	116.4	a	88.6	a	50.7	a	11275.2	a
Ranman	22.8	b	40.1	a	11.3	a	1248.8	a
Resist 57 Std	33.7	ab	70.1	a	423.8	a	415.4	a
Resist57	111.2	a	147.3	a	40.7	a	15140.1	a
Ridomil Gold Bravo	51.4	ab	86.7	a	3760.5	a	21620.8	a
Non-treated	117.4	a	38.7	a	1526.8	a	15481.7	a
Tuber DNA								
Heads up	556.5	a	6.9	a	58.8	a	24.7	a
Omega 500F	2708.2	a	2.4	a	15.7	ab	18.7	ab
Orondis Gold	1344.8	a	4.4	a	1.2	bc	1.6	b
Ranman	163.2	a	2.7	a	1.5	bc	5.9	ab
Resist 57 Std	1605.8	a	2.8	a	1.6	bc	41.8	a
Resist57	177.0	a	3.9	a	0.6	c	20.1	ab
Ridomil Gold Bravo	664.4	a	4.6	a	1.1	bc	5.9	ab
Non-treated	1119.0	a	5.3	a	6.2	abc	123.0	a

6.4.4 Inoculum test in stem and tuber from pink rot field trials

The homogeneity of variance test indicated data could not be combined, thus each field was analyzed separately (Table 6.9). None of the fungicide treatments (for DNA from stem and tuber) were significantly different from each other in each field (Table 6.10). Stem DNA from eight treatments was not significantly different (Table 6.10). Similarly, tuber DNA from eight treatments was not significantly different in both fields (Table 6.10). Although statistically insignificant, tubers from Orondis Gold and stems from Resist 57 treated plots had the least amount of DNA (Table 6.10). DNA from stem and tuber samples was higher in Field 113 than in Field 524.

Table 6.9 Analysis of variance of the main effects of *Phytophthora erythroseptica* DNA (pg/g of tissue) in stem and tuber from different fungicide treatment plots, combined and each field. Significance indicated by $p \leq 0.05$ (Tukey HSD).

Source	df	Sum of squares	F Ratio	Prob > F
Stem DNA pg/g of tissue				
Combined for all fields				
Treatment	7	9.9885	0.7795	0.6075
Field	1	13.58845	7.4232	0.009
Treatment*Field	7	9.4300	0.7359	0.6426
2018 (524)				
Treatment	7	4.1626	0.3010	0.9465
2018 (113)				
Treatment	7	16.0802	1.3613	0.2663
Tuber DNA pg/g of tissue				
Combined for all fields				
Treatment	7	5.1611	0.6737	0.6932
Field	1	2.4339	2.2241	0.1424
Treatment*Field	7	6.4960	0.8480	0.5537
Year 2018 (field 524)				
Treatment	7	7.7984	1.0590	0.4187
Year 2018 (field 113)				
Treatment	7	3.3438	0.4202	0.8801

Table 6.10 *Phytophthora erythroseptica* DNA in stem and tuber collected from two different fields (524 and 113), stem collected before vine kill and tubers at two cm diameter. Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Treatments	DNA from Stem				DNA from Tuber			
	Field 113		Field 524		Field 113		Field 524	
Heads up	346.1	a	190.2	a	621.4	a	451.6	a
Omega 500F	909.2	a	158.6	a	678.2	a	708.7	a
Orondis Gold	436.1	a	188.8	a	740.4	a	35.3	a
Ranman	679.0	a	183.1	a	755.4	a	296.7	a
Resist 57 Std	2646.0	a	77.2	a	941.6	a	749.0	a
Resist57	143.7	a	199.4	a	2801.9	a	459.3	a
Ridomil Gold Bravo	6813.4	a	885.1	a	640.7	a	386.4	a
Non-treated	29741.0	a	262.5	a	521.6	a	428.2	a

6.4.5 Inoculum test in the soil during the cropping season in Pythium leak trial

Statistical analysis showed a significant difference between years and sample collection timing, so the data from each timing were analyzed separately (years data not shown; Table 6.11). The sample collection timings were significantly different in all years and fields (Table 6.11). The samples collected from different treatments at each sample collection time were not significantly different (Table 6.12). The DNA in the soil collected from two fields was more or less uniform at each soil sample time (Fig 6.1). However, DNA in soil was significantly different at each soil sampling time. DNA from soil was significantly higher tested after inoculation and significantly different (Figure 6.2). The inoculation of the field trials had worked well because when tested before harvest, the DNA in the soil was still in good amount (Figure 6.2), which was enough to cause infection in the tubers. The *Pythium ultimum* DNA in the soil before inoculation was low and significantly different except in 2017 (Figure 6.2). In 2016, the DNA level in soil was very high, which can have been carried out to the following year because, in 2017, *P. ultimum* DNA was very high before inoculation (Figure 6.2).

Table 6.11 Summary of analysis of variance of the main effects of *Pythium ultimum* soil DNA (pg/g of soil) collected from different fungicide treatment plots at three different stages of potato growing season. (Three-sample timing combined in each year). Significance indicated by $p \leq 0.05$.

Source	DF	Sum of Squares	F Ratio	Prob > F
Year 2016, Field 524				
Treatment	7	8.1744	1.2125	0.3144
Timing	1	71.4025	74.1363	<.0001
Treatment*Timing	7	7.1075	1.0542	0.4071
Year 2017, Field 524				
Treatments	7	10.7885	2.8988	0.01
Timing	2	10.0571	9.4580	0.0002
Treatments*Timing	14	6.4689	0.8691	0.5942
Year 2018, Field 524				
Treatments	7	0.3452	1.3404	0.2441
Timing	2	30.2777	411.4867	<.0001
Treatments*Timing	14	0.4255	0.8260	0.639
Year 2018, Field 113				
Treatments	7	0.5831	0.5793	0.7706
Timing	2	31.3290	108.9250	<.0001
Treatments*Timing	14	0.9370	0.4654	0.9441

Table 6.12 Analysis of variance for the main effect of Soil DNA (pg/g of soil) collected from seven fungicides and one control treatment plot in 2016, 2017, 2018 (field 524 and 113). Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
2016				
Timing 1	7	12.6522	1.5572	0.1962
Timing 2	7	2.6297	0.4907	0.8319
2017				
Timing 1	7	9.2092	1.7961	0.1345
Timing 2	7	7.6287	1.3792	0.259
Timing 3	7	0.4194	0.8279	0.5744
2018 (524)				
Timing 1	7	0.5855	0.9871	0.4636
Timing 2	7	0.1380	1.0533	0.4221
Timing 3	7	0.0472	0.9743	0.472
2018 (113)				
Timing 1	7	0.7194	0.7915	0.6015
Timing 2	7	0.3941	0.2589	0.964
Timing 3	7	0.4067	0.6903	0.6793

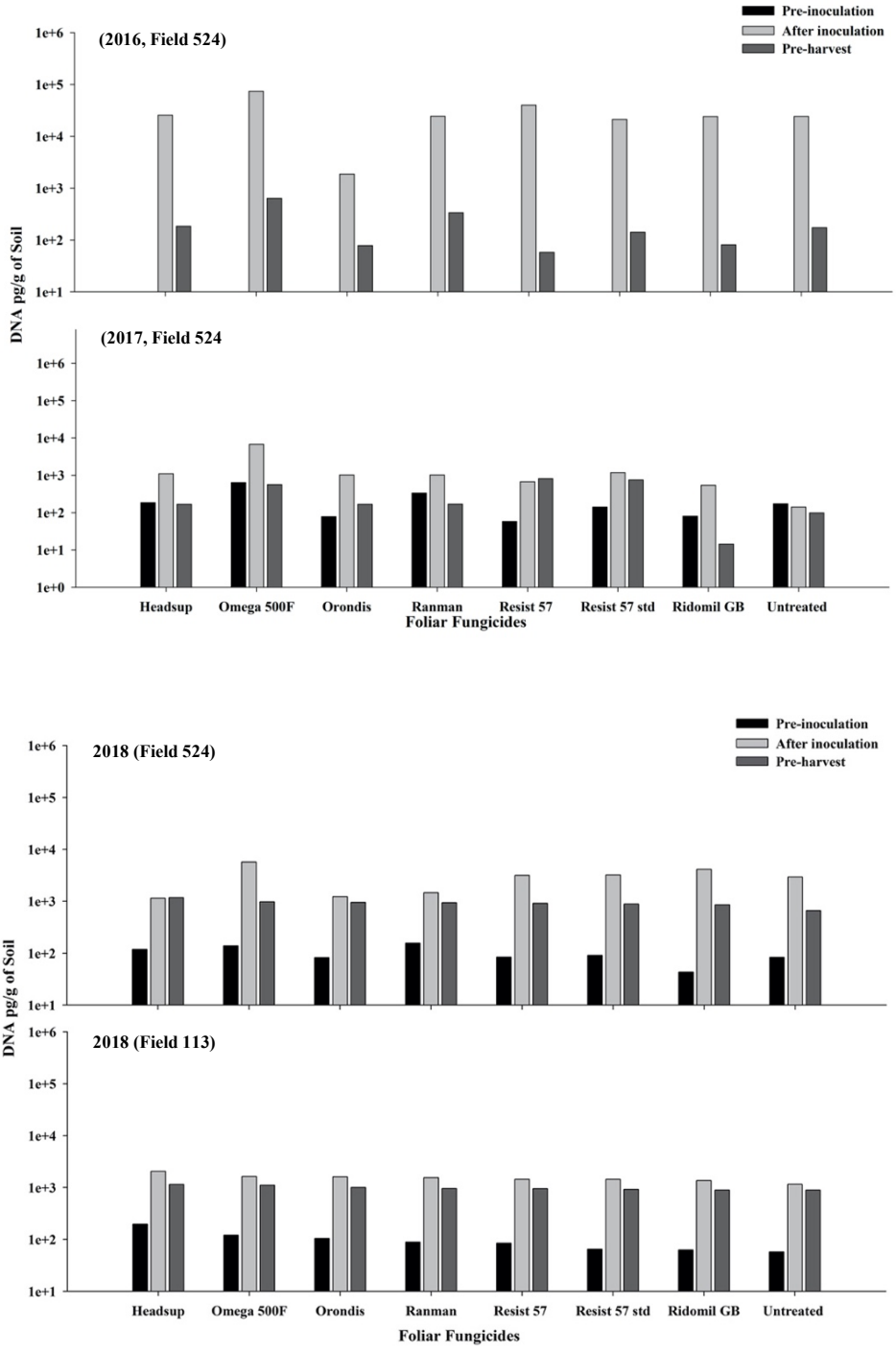


Figure 6.1 DNA (pg/g of soil) of *Pythium ultimum* in soil collected from two different fields at two to three different stages of potato growing season. The DNA level of *Pythium ultimum* in soil collected from the seven foliar spray and non-treated plots were not significantly different (Tukey HSD, $p \leq 0.05$).

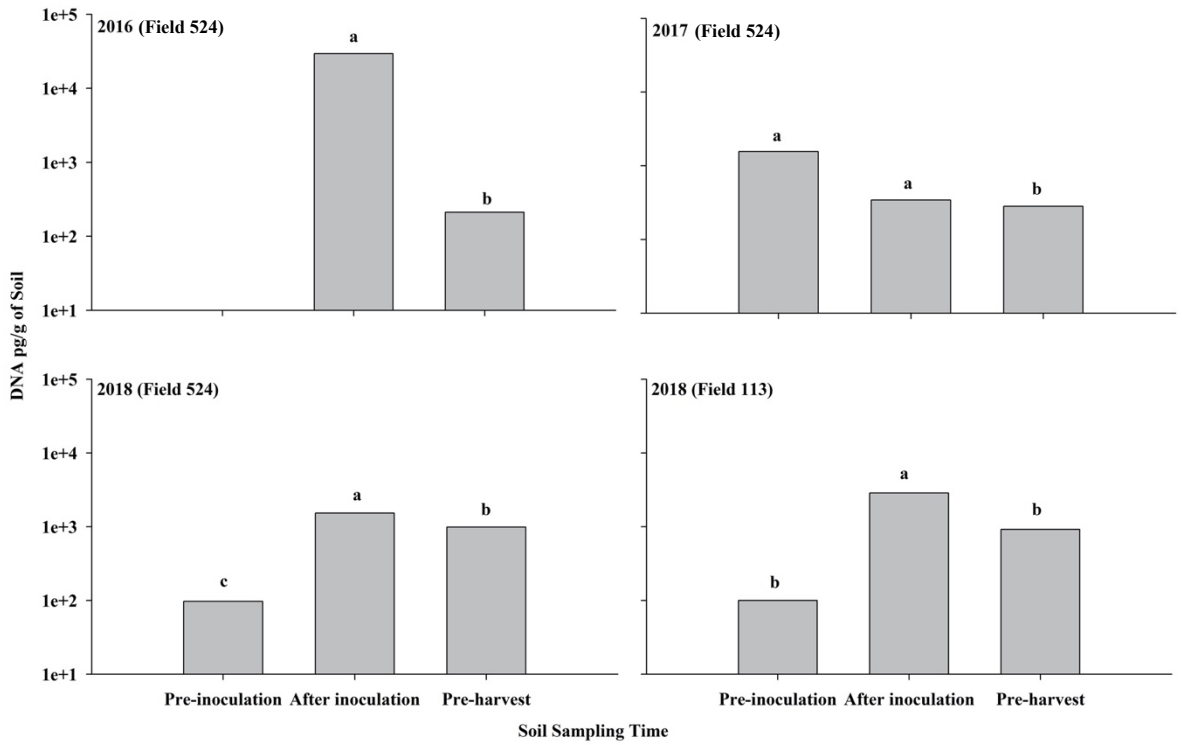


Figure 6.2 DNA (pg/g of soil) of *Pythium ultimum* in soil collected from 524 and 113 fields at two (2016) to three different stages of potato growing season. Data were collected in 2016 (field 524), 2017 (field 524) and 2018 (field 524 and 113). The DNA level of *Pythium ultimum* in the soil collected from the seven foliar spray and non-treated plots was significantly different (Tukey HSD, $p \leq 0.05$).

6.4.6 **Inoculum test in the soil during the cropping season in pink rot trial**

Statistical analysis showed a significant difference between fields and sample collection timing, so the data from each timing were analyzed separately (combined field data not shown; Table 6.13). The sample collection timings were significantly different in both fields (Table 6.13). The samples collected from different treatments at each sample collection time were not significantly different (Table 6.13). The DNA in the soil collected from different fields was uniform at each soil sample time (Fig 6.3). However, DNA in soil was significantly different at each soil sampling time. DNA from soil was significantly higher at a pre-harvest stage in field 113 (Figure 6.4). The DNA in the soil was in good amount and enough to cause infection in the tubers (Figure 6.4). The *Phytophthora erythroseptica* DNA in the soil before inoculation was low and significantly increased after-inoculation to the pre-harvest stage (Figure 6.4). The DNA level in soil was more uniform in field 524 at each stage of inoculation compared to 113 (Figure 6.3).

Table 6.13 Analysis of variance for the effects of *soil Phytophthora erythroseptica* DNA (pg/g of soil) collected from different fungicide treatments, combined and each field. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
Year 2018, Field 524				
Treatment	7	5.6445	1.1857	0.3219
Timing	2	103.3133	75.9607	<.0001
Treatment*Timing	14	10.3313	1.0852	0.3854
Year 2018, Field 113				
Treatment	7	1.8021	0.6171	0.7401
Timing	2	62.4981	74.9076	<.0001
Treatment*Timing	14	4.5316	0.7759	0.691
Year 2018, Field 524				
Timing 1	7	4.0590	0.7226	0.6542
Timing 2	7	1.5672	0.9016	0.5213
Timing 3	7	10.3495	1.4944	0.2165
Year 2018, Field 113				
Timing 1	7	0.5231	1.5059	0.2127
Timing 2	7	4.7700	0.6506	0.7104
Timing 3	7	1.0406	0.9622	0.48

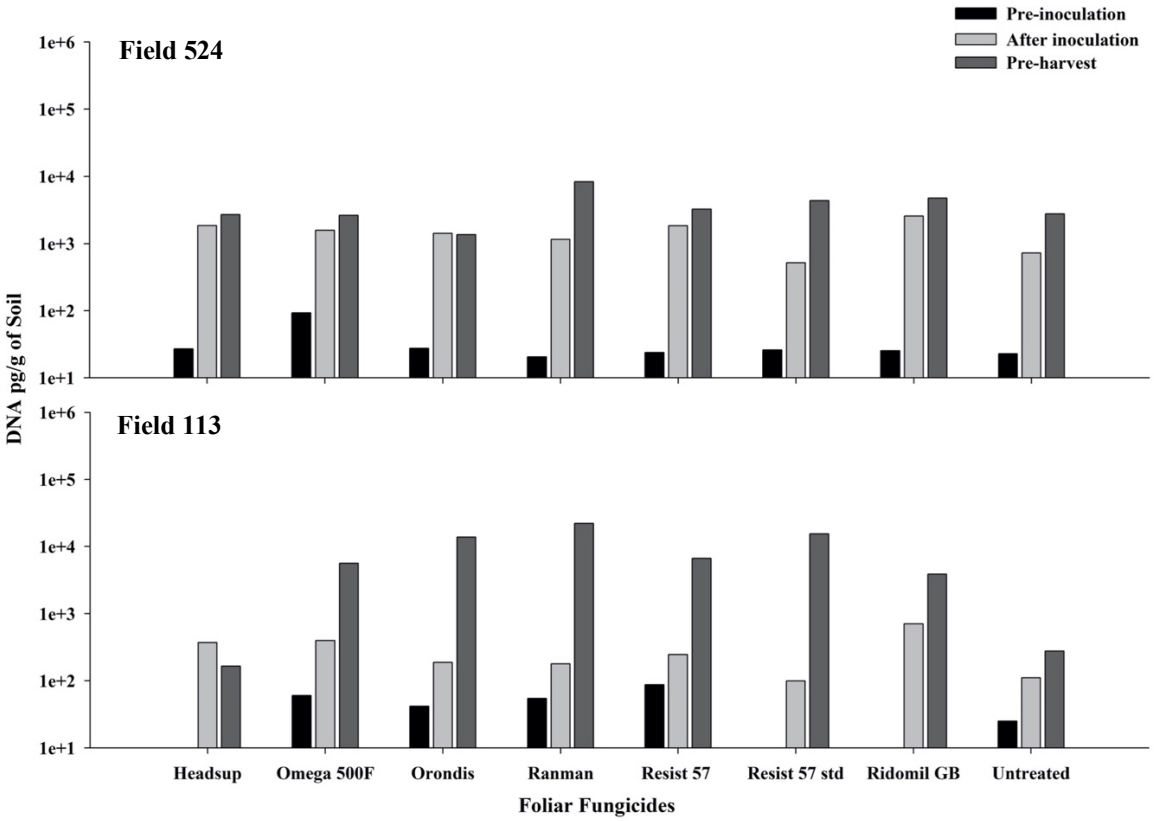


Figure 6.3 DNA (pg/g of soil) of *Phytophthora erythroseptica* in soil collected from two different fields at three different stages of potato growing season. The DNA level of *Phytophthora erythroseptica* in soil collected from the seven foliar spray and non-treated plots was significantly different at different stages of inoculation (Tukey HSD, $p \leq 0.05$). Data were collected from two different fields (524 and 113). The top graph is for field 524, and the bottom is for field 113.

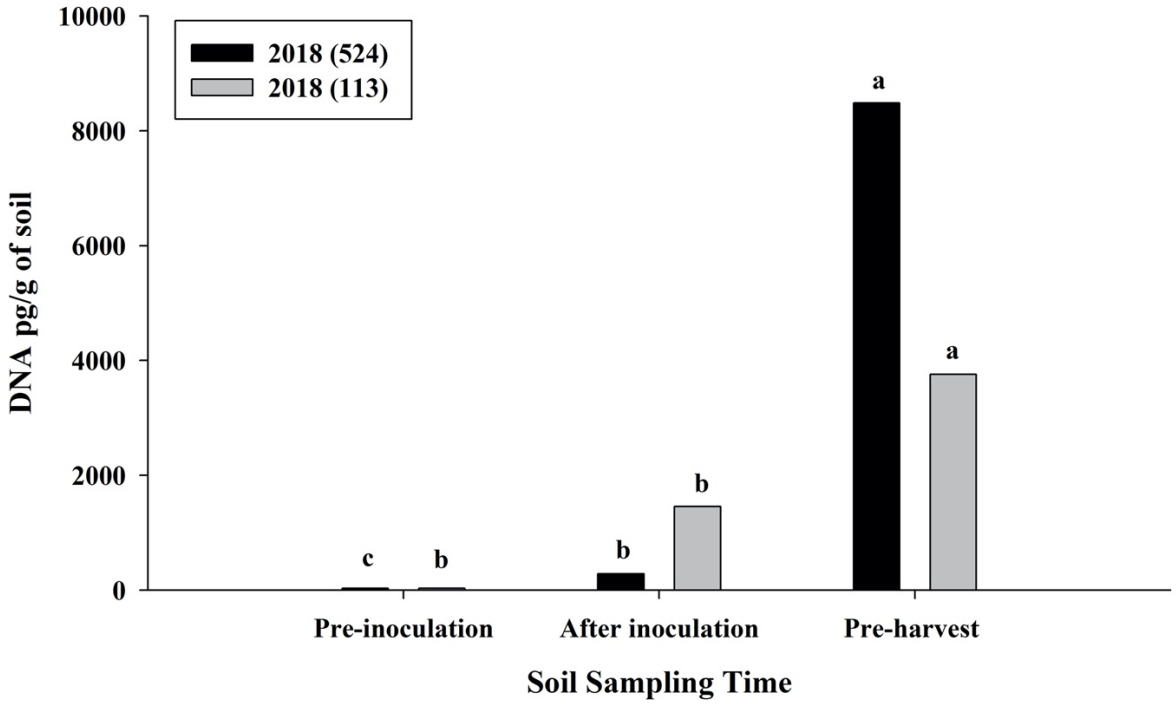


Figure 6.4 DNA (pg/g of soil) of *Phytophthora erythroseptica* in soil collected from two different fields at three different stages of potato growing season. The DNA level of *Phytophthora erythroseptica* in soil collected from three stages of inoculation was significantly different (Tukey HSD, $p \leq 0.05$). Data were collected from two different fields (524 and 113).

6.4.7 Yield from Pythium leak and pink rot trails

The yield from the Pythium leak trial was significantly different between years. However, each year's yield from foliar in-season fungicide treatments was not significantly different except in 2018 (field 118; Table 6.14). Yield from the Pythium leak trial was almost uniform in three years from the same field for each treatment (524; Figure 6.5). In 2017 and 2018 (field 524), the yield was low in Resist 57 and Ridomil Gold Bravo treatments. However, these treatments were not significantly different from other treatments in that year. The foliar application of in-season fungicide did not considerably increase the yield in the Pythium leak trial (Figure 6.5).

Statistical analysis showed a significant difference in yield between the fields in the pink rot trails (Table 6.15). Field 113 had a significantly higher yield than field 524 (Table 6.16). The yield from in-season foliar treatments in each field was not significantly different (Table 6.15). In 2018, the yield from each treatment was similar except for Resist 57 and Resist 57 standards (Figure 6.6). The yield from Resist 57 and Resist 57 standards was low in field 527; however, the yield from these two treatments was not significantly different from other treatments, including the non-treated control.

Table 6.14 Analysis of variance for the main effects of the yield (kg/ha) harvested from different fungicide treatment plots of Pythium experiment, combined and each year. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
Combined for all years				
Treatment	7	443799030	2.3969	0.0265
Year	3	4826417424	60.8221	<.0001
Treatment*Year	21	921500999	1.6590	0.0516
2016				
Treatment	7	108675785	0.4728	0.8519
2017				
Treatment	7	170988984	0.9619	0.4641
2018 (524)				
Treatment	7	423095872	2.6516	0.0155
2018 (113)				
Treatment	7	508743078	0.8553	0.5451

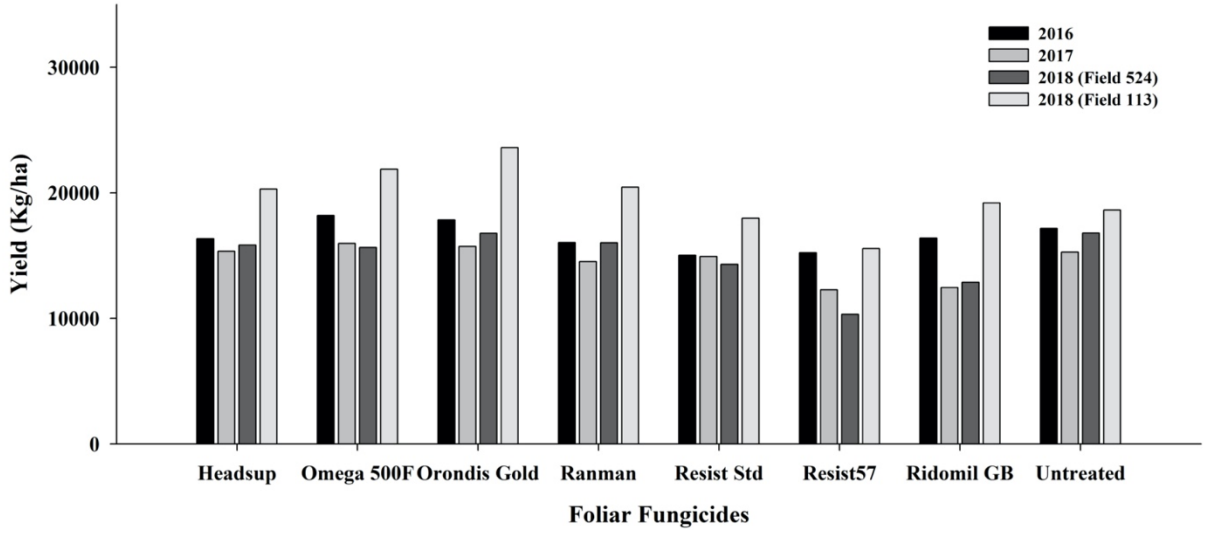


Figure 6.5 Total yield (kg/ha) harvested from two different fields and eight different fungicide treatments of *Pythium ultimum* experiment. The yields between the foliar fungicide treatment were not significantly different (Tukey HSD, $p \leq 0.05$).

Table 6.15 Analysis of variance for the main effects of the yield (kg/ha) harvested from different fungicide treatment plots of pink rot experiment, combined and each field. Significance indicated by $p \leq 0.05$.

Source	df	Sum of squares	F Ratio	Prob > F
Combined for all years				
Treatment	7	1055756118	2.5042	0.0281
Field	1	1347093014	22.3667	<.0001
Treatment*Field	7	348760657	0.8272	0.5698
2018 (field 524)				
Treatment	7	509412558	2.3147	0.0592
2018 (field 113)				
Treatment	7	1021992343	1.7329	0.1487

Table 6.16 Least square means table of yield (kg/ha) harvested from two different *Phytophthora erythroseptica* fungicide experiment fields (524 and 113). Least square means within columns followed by the same letters do not differ, based on multiple comparison Tukey HSD ($\alpha \leq 0.05$).

Field	Least Sq Mean	
113	32612.0	a
524	23398.2	b

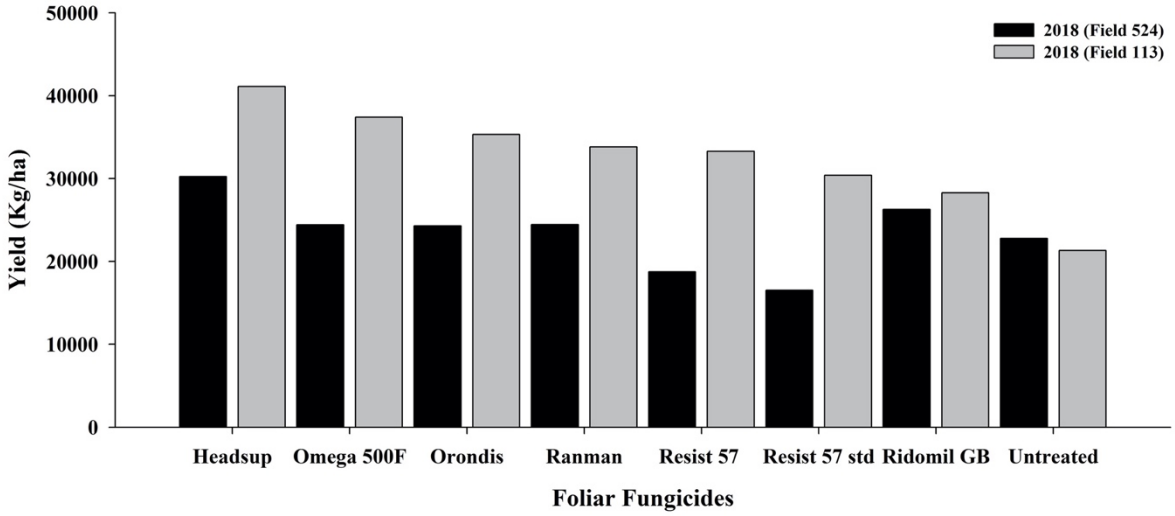


Figure 6.6 Total yield (kg/ha) harvested from two different fields and eight different fungicide treatments of *Phytophthora erythroseptica* experiment. The yields between the foliar fungicide treatments were not significantly different (Tukey HSD, $p \leq 0.05$).

6.5 Discussion

Many diseases challenge the potato tubers from planting to storage. The disease-free potato tubers are crucial to breaking the disease cycle during the growing season to the storage. In addition, any contaminated or diseased tubers stored even in well-equipped storage cannot regain their original status. So, well-facilitated storage can only maintain the health of potatoes. So, growers' priorities are to grow healthy potatoes, reduce injuries, maintain health during the harvest and handling operations and store them healthy. However, many pathogens can infect the potatoes without injuries, such as *Phytophthora erythroseptica*, *P. infestans,* etc. Previous studies have shown that *Phytophthora erythroseptica* and *P. ultimum* can be controlled with in-season foliar application of systemic fungicides. There are very limited fungicides to control the Pythium leak and pink rot in the storage. So, our study's main objective was to evaluate the commercial in-season systemic foliar fungicides to see their effect in controlling these diseases in storage.

Systemic fungicides are more effective than other contact fungicides because the systemic fungicides can translocate to different parts of the plants. Mefenoxam is a systemic fungicide used for many years to control *P. ultimum* and *P. erythroseptica*. In this study, mefenoxam was ineffective in controlling the Pythium leak and pink rot in the challenge inoculation study. It has been reported that foliar application of mefenoxam might not be as effective as in-furrow application as seen in some experiments (Taylor, Salas & Gudmestad, 2004; Tsror et al, 2021). The in-furrow application of mefenoxam sometimes has disadvantages. Several environmental factors are reported to affect the efficacy of mefenoxam in the field (Mulrooney & Gregory, 2002; Peters et al, 2003). The soil moisture level directly impacts the effectiveness of the mefenoxam (Mulrooney & Gregory, 2002; Peters et al, 2003). However, in our study, we used mefenoxam as a foliar spray. Also, the bioactivity of mefenoxam in potato tubers has been reported to reduce overtime (Bruin, Edgington & Ripley, 1982). We used potato tubers stored at 12.8°C for two to three months after harvest in all our challenge inoculation trials. It has been reported that the mefenoxam was more concentrated in or near the periderm of the tuber (Bruin, Edgington & Ripley, 1982). So, any deep wound created on the tuber surface during harvest and handling operation can let *P. ultimum* and *P. erythroseptica* easily pass this chemical along with the physical barrier. In our challenge inoculum study, potato tubers were bruised by dropping them from a three-meter

height in a metal bruiser. The bruising had created a fresh deep wound favorable to *P. ultimum* and *P. erythroseptica*. In addition, the isolates of *P. ultimum* and *P. erythroseptica* resistant to mefenoxam have evolved and been reported in many potato production areas (Taylor et al., 2002). In our study, we had used mefenoxam sensitive isolates of both *P. ultimum* and *P. erythroseptica*. However, we did not isolate and test the isolates from the field. The continuous potato production and application of mefenoxam every year might have changed the pathogen population in the soil from sensitive to insensitive to mefenoxam. So, the high effectiveness of mefenoxam may be achieved in areas where there are no mefenoxam insensitive isolates and if the tubers are harvested with minimum damage in the periderm.

Mono- and di-potassium phosphite (Resist 57) is a systemic fungicide labeled to control many diseases of crops, including Phytophthora and Pythium leak in potatoes. So, phosphite is a phosphorous acid-based fungicide. In our study, both two or three in-season foliar sprays of phosphite had reduced the Pythium leak and pink rot in the challenge inoculation study. Phosphite has been reported to effectively control several oomycete pathogens (Cooke & Little, 2002). The phosphorous acid-based fungicide was very effective post-harvest pre-storage treatment for *P. erythroseptica* (Johnson, Inglis & Miller, 2004; Miller et al, 2006). However, phosphorous acid was less effective in controlling Pythium leak (Johnson, Inglis & Miller, 2004). Phosphite had consistently reduced the Pythium leak in our challenge inoculation study. However, the results of some years were not significantly different from other fungicides and non-treated control. Phosphite was very effective in controlling pink rot, as described by many other studies (Johnson, Inglis & Miller, 2004; Miller et al, 2006). Phosphite can provide consistent performance against pink rot and has been reported to control pink rot when applied even after infection (Miller et al, 2006; Taylor, Pasche & Gudmestad, 2011).

Oxathiapiprolin (Orondis) is a systemic fungicide with the active ingredient of piperidinyl-thiazole-isoxazoline (Pasteris et al, 2016). Oxathiapiprolin has been used to control oomycetes like *Pseudoperonospora humuli* in hops and *P. erythroseptica* in potatoes (Ge et al, 2019; Higgins, Miles & Hausbeck, 2021). Orondis Gold is a mixture of Oxathiapiprolin and mefenoxam. Since Oxathiapiprolin is a high-risk fungicide, it has been used with a mix of other systemic fungicides to avoid resistance build-up (Cohen, Rubin & Galperin, 2018b; Miao et al, 2016). The most effective combination of Oxathiapiprolin has

been with mefenoxam because it has enhanced efficacy when mixed with mefenoxam (Cohen, Rubin & Galperin, 2018a; 2018b). It has been reported that even a small quantity of oxathiapiprolin absorbed by the plant's root system is effective in controlling the foliar oomycete pathogens (Cohen, Rubin & Galperin, 2018a). Orondis Gold has been reported to effectively control plant pathogenic oomycetes, including *P. erythroseptica* but not the *Pythium species* (Ge et al, 2019; Pasteris et al, 2016). Orondis Gold was very effective in controlling pink rot even in the presence of mefenoxam resistant isolates of *P. erythroseptica* (Ge et al., 2019; Xuemei, 2016). In our challenge inoculation study, Orondis Gold effectively controlled *P. erythroseptica*. However, ineffective in controlling *Pythium ultimum*. Our result agreed with the findings as reported by previous researchers (Ge et al., 2019; Pasteris et al, 2016; Xuemei, 2016).

Cyazofamid (Ranman) is another systemic fungicide that has been used to effectively control oomycete pathogen in many crops, including pink rot in some locations (Higgins, Miles & Hausbeck, 2021; Gundersen & Inglis, 2005). The foliar application of cyazofamid was reported to control the potato tubers from *P. infestans* effectively. In our study, cyazofamid was used as a foliar spray and did not significantly reduce Pythium leak. However, cyazofamid significantly reduced disease severity in potato tubers challenge inoculated with *P. erythroseptica.* Cyazofamid was very selective in controlling oomycete pathogens (Mitani et al, 2001b). It had very high antifungal activity against *Phytophthora* species such as *Phytophthora infestans*, *P. sojae*, *Pythium sylvaticum*, *P. aphanidermatum*, *Pythium spinosum* (Mitani et al, 2001a; 2001b). The inability of cyazofamid to control *P. ultimum* in our study is unknown.

Headsup is a bio fungicide, an extract of *Chenopodium quinoa* saponins. *C. quinoa* saponins have been used in many crops as a foliar crop protectant to control the foliar, seed, and soilborne diseases (AI-Mughrabi, Vikram & Poirier, 2010; Kandel et al, 2019; Stuardo & San Martin, 2008). In a study, the foliar spray and seed treatment with *C. quinoa* saponins effectively controlled common scab, dry rot, and black scurf of potato (AI-Mughrabi, Vikram & Poirier, 2010). In another study, *C. quinoa* saponins were modified with alkali and were very effective in inhibiting Botrytis cinerea's mycelial growth and germination (Stuardo & San Martin, 2008). In our study, *C. quinoa* saponins did not significantly reduce disease severity and incidence in Pythium leak and pink rot.

Fluazinam (Omega 500F) is a systemic fungicide with a multi-site mode of action and less fungicide resistance risk. It has been used in several studies to control different plant pathogens, including potato pathogens such as *P. infestans* (Schepers et al, 2018). In this study, Fluazinam significantly reduced the disease severity in pink rot. Fluazinam did not control Pythium leak in our challenge inoculation study. The residue of fluazinam in potato tuber, stem, and soil was reported to last only for a few days (Feng et al, 2015). So, fluazinam might have full potential to control pathogens during potato growing season but the limited potential to inhibit the pathogens in storage.

The pathogens present in potato and the level of inoculum in the inoculated soil was monitored throughout the study. This is very important, particularly to ensure the inoculation was successful and pathogens had infected the plant. In this study, we had used high inoculum density in the field and very susceptible cultivar (Russet Burbank) to *P. ultimum* and *P. erythroseptica*. In the challenge inoculation study, the bruising method we had used produced deep and multiple wounds and 16 hours of inoculation allowed the pathogen to infect the potato tissue. Overall, our experiments were conducted at a very high inoculum pressure in the field and lab. So, our results may underestimate the potential of fungicides to control or prevent the pathogens in natural conditions.

In this study, the in-season foliar fungicides were not phytotoxic. However, some plots treated with Resist 57 had some yellow plant symptoms. The recurrent symptoms should be tested for any phytotoxicity. Surprisingly, in our study, the yield from Resist 57 treated plot was lower than that of other treatments and non-treated control, however they were not statistically significant. The low yield in plots treated with Resist 57 is unknown but could be related to the plant symptoms.

7 SUMMARY

This dissertation attempted to improve the in-depth understanding of *P. infestans* genotypes and population diversity in two developing countries, Bangladesh, and Indonesia. Before this study, very little was known about *P. infestans'* genotypic composition and population diversity in Bangladesh and Indonesia. Since the population of *P. infestans* is dynamic and many metalaxyl resistant genotypes are evolving, breeding cultivars resistant to late blight is the best strategy to combat late blight in developing countries where modern technologies are lacking. Breeding cultivars of potato resistance to late blight using the bioengineer technique has many advantages over conventional breeding, mainly the short duration to develop late blight resistance potato cultivars. Thus, The Feed the Future Biotechnology Potato Partnership was formed in 2015 as a 5-year, $6 million multi-institutional cooperative agreement with USAID to introduce bioengineered potato products in farmer- and consumer-preferred cultivars into Bangladesh and Indonesia. These bioengineered potatoes will have 3 R-gene resistance to late blight. Based on SSR analysis, mating type test, and mitochondrial haplotype test, we revealed that the Indonesian *P. infestans* population was dominated by EU_2_A1, some other unique genotypes, and the Bangladeshi *P. infestans* population was dominated by mainly large sub-clonal variation of EU_13_A2. It is very important to study and understand the changes in population diversity to update management strategy accordingly. These findings will serve as a baseline for future population diversity studies and inform the development of integrated strategies to extend the efficacy and durability of the USAID FtFBPP 3 R-gene potato cultivars developed for release in Bangladesh and Indonesia.

Furthermore, growers now know what genotypes they have in their locality and can decide what type of fungicides they need to spray. In addition, the knowledge of the diversity of *P. infestans*, virulence factors of the genotypes/clonal lineages, gene flow, and mode of reproduction is very useful in tracking the origin and evolution potential of the pathogen (Cooke and Lees 2004; Zwankhuizen et al. 1998, 2000). Further work can be done to determine the virulence genes in the isolates/genotypes of the Bangladeshi and Indonesian *P. infestans* population. Also, the effect of host selection on genotypes of *P. infestans* can be studied in detail because these two countries have been producing late blight susceptible potato cultivars for a long time.

Late blight can destroy the potato fields in a few days under conducive environmental conditions if proper control measures are not applied. Early, accurate and rapid identification of pathogens allows growers enough time to make appropriate decisions on best management strategies (De Boer and Lopez 2012; DeShields et al. 2018). However, few diagnostic assays can detect pathogens in a very short time. Therefore, timely and accurate diagnosis and characterization of newly emerging or re-emerging pathogens by utilizing molecular-based technologies can contribute to the development or modification of new disease management strategies. Molecular techniques such as polymerase chain reaction (PCR), quantitative real-time PCR (qPCR), sequencing, and loop-mediated isothermal amplification (LAMP) are technologies that can be used to detect pathogens in the host even before the onset of the first visible symptoms. In this dissertation, we developed species-specific LAMP primer sets to detect *P. infestans* in their early stages. These LAMP assays coupled with Genie II/III C instruments (OptiGene Limited, Blatchford Rd, Horsham UK) can detect *P. infestans* as early as 7-8 min, and the test can be carried on-site (in the field). In the future, further work can be done to develop primers that can detect *P. infestans* at the genotype level. Furthermore, different experiments can be conducted to improve the stem primers to make the LAMP primers even more sensitive and specific.

Potatoes are kept in storage for a few weeks to several months after harvest based on the intention of use or market availability (Guenthner, 1999). *Pythium ultimum* infected potato tuber may rot or contaminate the whole lot in storage, causing significant economic loss. Several factors affect the disease incidence and severity of Pythium leak in storage. In this dissertation, we studied the four pre-storage temperatures (15°C, 20°C, 25°C, and 30°C) and two storage temperatures (to emulate the condition in the grower's field. And the tubers after incubated for 48h at those pre-storages (8.8°C and 12.8°C). We determined the tubers incubated at 15°C and stored at 8.8°C had minimum disease severity and incidence followed by 20°C.

Similarly, disease severity and incidence were lower in tubers incubated at 15°C and stored at 12.8°C. In artificially inoculated experiments, tubers showed symptoms much earlier than the naturally infected tubers; however, the trend of disease development over time was similar. We also determined that a single oospore is enough to start the disease when conducive temperature and moisture are maintained. These findings will help to develop a

disease forecasting model. More factors such as cultivars, different moisture levels at harvest, different soil types, and different types of harvesters can be studied to see any effect of these factors on disease severity and incidence in storage.

Growers are recommended to practice integrated disease management strategies to control *P. erythroseptica* and *P. ultimum.* As part of an integrated disease management strategy, growers rely on fungicides to combat diseases. However, there are no effective fungicides that can be applied in storage to control storage rot diseases. Therefore, growers must use in-season foliar fungicides to eliminate or reduce pathogen inoculum levels. There are commercially available fungicides on the market that can be used during the growing season to control storage rot diseases such as Pythium leak and pink rot. However, these foliar fungicides need to be evaluated for each pathogen before recommending them to the growers. Therefore, we evaluated seven commercially available fungicides to determine their effectiveness in reducing disease in storage. We found out that the mono- and di-potassium phosphite effectively controlled *P. erythroseptica* and *P. ultimum* in storage. In addition, Cyazofamid, Fluazinam, Oxathiapiprolin, and Mefenoxam significantly reduced the pink rot in storage. Therefore, the findings of this study can be added to an integrated disease management strategy to minimize the loss in storage. In the future, the timing of the last spray in season can be moved very close to the harvest and see how moving the last spray close to harvest affects the disease severity in storage. Also, it would be better to test the additive effects of some fungicides that reduced the disease severity in storage.

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Appendix

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ORIGINAL ARTICLE

Plant Pathology WILEY

Genotypic and phenotypic characterization of *Phytophthora infestans* populations on Java, Indonesia

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Abstract

Phytophthora infestans is endemic to Indonesia and can infect potato crops at any stage in the growing season. Little is known about *P. infestans* populations in Indonesia. The objectives of this study were first to identify the genotypes causing late blight in the main potato-growing regions on Java in Indonesia, and secondly to examine genotypic diversity in the *P. infestans* populations in those regions. Samples were collected on FTA cards (n = 140) or in tubers (n = 6) from 15 locations in nine regencies over four years (2016–2019). Microsatellite analysis revealed that late blight outbreaks in these regencies were caused by EU_2_A1 and other genotypes that are unique to Indonesia. Eighty percent of the samples that amplified with CAPS markers were the A1 mating type. Cultures of six isolates were determined to be the A1 mating type based on the pairing test, and of these, two isolates were intermediate and four were sensitive to metalaxyl-M (mefenoxam). The mode of reproduction of the *P. infestans* population on Java, Indonesia, was found to be clonal. However, as the sample size in this study was small, more isolates need to be tested to confirm this. Microsatellite analysis revealed that 90% of Indonesian samples had trisomic loci. A high number of multilocus genotypes (MLGs) were found in all nine regencies (131 MLGs out of 146 samples). Results indicate that there is ongoing polyploidization in these populations due to a high mutation rate and no selection pressure from the susceptible potato hosts that are being grown in Indonesia.

KEYWORDS
FTA cards, genetic structure, late blight, microsatellite analysis, pathogen, potato

1 | INTRODUCTION

Phytophthora infestans is recognized as one of the most destructive pathogens of potato, tomato, and other solanaceous crops (Fry, 2008). The pathogen causes late blight of potato, which is a major constraint of profitable potato production worldwide (Fry, 2008). Late blight management costs and losses from yield reductions are estimated at over $6 billion a year (Haverkort *et al*., 2008).

Populations of *P. infestans* are dynamic, with mutation, migration, sexual reproduction, and host having contributed to the evolution of new clonal lineages or genotypes with different epidemiological, genotypic, and phenotypic characteristics (Goodwin, 1997; Hu *et al*., 2012). Globalization has increased the trade in agricultural commodities such as potato between countries. Potato tubers intended for processing or for use as seed are imported or exported from one country to another based on supply and demand. *P. infestans* remains dormant (latent) in seed tubers at low temperatures (4°C). As such, symptomless seed tubers are easily overlooked in intercontinental shipments (Johnson and Cummings, 2009). The introduction of new genotypes in seed tubers has resulted in changes in local population compositions of

P. infestans (Goodwin, 1997). Because the evolution of *P. infestans* is rapid, it has been a challenge to manage the pathogen even in developed countries where modern technologies such as disease and weather forecasting systems, modern fungicide chemistries, and disease resistant cultivars are available. As such, in developing countries like Indonesia, late blight is a significant impediment to profitable potato production.

Indonesia is one of the largest potato producers in Southeast Asia (FAO, 2008). It is believed that the Dutch East India Company first introduced potatoes to West Java around 1795 (FAO, 2008). Since then, potatoes have been grown in Indonesia by small-scale farmers throughout the archipelago, mainly in the highlands (800–1,800 m a.s.l.; FAO, 2008). Potato production in Indonesia has increased significantly since 1969, mainly due to an increase in the total production area and to a lesser extent to increases in productivity (Adiyoga *et al*., 1999). In Indonesia, more than 50% of potatoes are grown on the island of Java, with 37% of those grown in West Java. In 2003, the average production per hectare was as high as 25–28 t/ha (Adiyoga, 2009). Potato cultivar Granola was bred and released in Germany in the late 1970s and later imported to Indonesia in the early 1980s (Adiyoga, 2009). In Indonesia this cultivar is prized for its medium to high yield potential, early maturity, and adaptation to the Indonesian climate. As a result, Granola is the most widely grown potato cultivar in Indonesia, accounting for 90% of production, and is grown mainly for the fresh market (Adiyoga *et al*., 1999; Adiyoga, 2009). However, Granola is susceptible to late blight (Adiyoga, 2009).

There are several factors that impact profitable potato production in Indonesia. Among them, late blight is one of the most important due to its impact on crop yield. Yield reductions may be as high as 90% depending on the growth stage of the crop when a disease outbreak occurs (Kusmana, 2003). Potato-growing regions in Indonesia, such as Sumatra and Java, have conducive environmental conditions for late blight year-round and *P. infestans* is endemic to these regions (Adiyoga, 2009). Infection can occur at any point in the potato crop growth cycle as soon as there is green plant tissue that can be infected. Thus, growers in Indonesia rely heavily on fungicides to control late blight, applying as many as 20–30 fungicide sprays in a single potato-growing season (Adiyoga, 2009). The best way to overcome the problem of late blight in Indonesia would be to develop a late blight-resistant potato cultivar. Breeding late blight resistance in potato cultivars has significant benefits due to the reduced cost of production through lower fungicide costs, reduced farmer exposure to fungicides, and reduced fungicide residues in food, land, and wastewater (Kirk *et al*., 2001).

Conventional breeding for resistance may take more than 10 years to produce a new resistant cultivar and by the time the new variety is released it may no longer be resistant, as local strains of the pathogen may have changed. The Feed the Future Biotechnology Potato Partnership was formed in 2015 as a 5-year, $6 million multi-institutional cooperative agreement with USAID to introduce bioengineered potato products in farmer- and consumer-preferred cultivars into Indonesia and Bangladesh. These bioengineered potatoes will have 3 *R*-gene resistance to late blight. These genes have been evaluated against *P. infestans* strains from Europe and the USA but have not been evaluated against Indonesian isolates. Thus, the objectives of this study were to identify the genotypes causing late blight in the main potato-growing regions of Java in Indonesia and to examine genotypic diversity in the *P. infestans* populations in the same regions. Because very little is known about the *P. infestans* populations in Indonesia, this information is critical to ensure the effectiveness and durability of any 3 *R*-gene cultivars that are released.

2 | MATERIALS AND METHODS

2.1 | Collection of *P. infestans* DNA samples and isolates

In this study, 146 samples were collected from 15 locations in nine regencies (second level administrative divisions equivalent to a municipality) in the major potato-growing areas of Java, Indonesia, from 2016 to 2019 (Table 1). DNA samples (140) were collected on Whatman Flinders Technology Associates (FTA) cards using the protocol suggested by the manufacturer (Sigma-Aldrich). Briefly, lesions on infected leaves were placed on top of the cellulose matrix on the FTA card, the paper flap was closed over the leaf, and using the blunt end of a pen, pressure was applied by rubbing to force juice out of the leaf, thus depositing DNA into the specialized matrix. The cards were then allowed to air dry for 30 min at room temperature, after which time they were suitable for long-term storage or shipment. Thirty tubers with infected leaves sandwiched between the two halves were shipped from Indonesia to Aberdeen, ID, USA. Unfortunately, due to delays in transit most of the tubers had started to decay by the time they were received, and only six separate isolates were obtained from the infected leaves. FTA cards were stored in a dry place at room temperature until processed for analysis. For pure cultures, actively growing hyphal tips or single spores were transferred to pea agar amended with RAN (rifamycin, 75 mg/L; ampicillin, 100 mg/L; and nystatin, 75 mg/L; Forbes, 1997). Details about the location and number of samples collected each year are listed in Figure 1 and Table 1.

2.2 | DNA extraction/purification of FTA card samples

FTA cards were processed in two different ways, either by purification of FTA cards using Whatman FTA purification reagent (GE Healthcare UK) or extraction of DNA from FTA cards using QIAmp DNA Investigator kit (Qiagen). The manufacturer's protocol was used to purify or extract the DNA from FTA cards. If insufficient DNA was extracted using QIAmp DNA Investigator kit, Whatman FTA purification reagent was used to purify the samples and a single FTA punch was used in PCR. DNA from isolates was extracted using DNeasy Plant Mini kit following the manufacturer's protocol (Qiagen).

TABLE 1 Geographical information on *Phytophthora infestans* samples (isolates and DNA samples on FTA cards) collected in different years from Java, Indonesia, and standard isolates used in the study

Region or country	Location	Total	DNA samples or isolates and collection year
Bandung	Pangalengan	32	DNA samples = 28, 2016; isolates = 4, 2019
Batu	Tutungrejo	9	DNA samples = 4, 2018; 5, 2019
Garut	Cisurupan	6	DNA samples, 2016
Majalengka	Argalingga	6	DNA samples, 2016
Mojokerto	Kebonaga	12	DNA samples = 5, 2018; 7, 2019
Pasuruan	Sedaeng, Tosari, Wonokitri, Ngadiwono	25	DNA samples = 11, 2018; 13, 2019; isolate = 1, 2019
Temanggung	Kledung	10	DNA samples = 8, 2018; 2, 2019
West Bandung	Lembang	10	DNA samples, 2016
Wonosobo	Serang, Parikesit, Tieng, Dieng	36	DNA samples = 25, 2018; 10, 2019; isolate = 1, 2019
USA	Michigan and Idaho	10	Isolates (year = 2008–2015; genotypes = US-8, US-22, US-23, and US-24)
UK	NA	2	Isolates from Michigan State University Kirk laboratory collection (year = 2004; genotypes = EU_6_A1, EU_13_A2)

Note: All Indonesian DNA samples and isolates were collected from local subsistence farmers' fields (multiple fields). Potato cultivar is Granola (seed produced in Indonesia), and crop rotation is potato–carrot/cabbage; fungicide spray (if any) is chlorothalonil- and/or mancozeb-based fungicides two to three times per week for 12 weeks. NA, not available

2.3 | Mating type determination

Cleaved amplified polymorphism sequence (CAPS) markers developed by Judelson *et al.* (1995) were used to determine mating types of *P. infestans* samples (n = 146) using standard PCR. The PCR products were cleaved using restriction enzyme *Hae*III and run on a 2% agarose gel for 90 min in a gel electrophoresis chamber (0.5 × TBE), stained with Gel Red (Biotium Inc.) and visualized with a Gel Doc EZ imager (Bio-rad). Samples with bands of 550 and 600 bp were determined to be A1 mating type, whereas samples with only a 600 bp band were determined to be A2 mating type. Results were cross-checked with AFLP molecular markers developed by Kim and Lee (2002).

Pairing tests were done for six isolates as described by Forbes (1997). Circular disks (5 mm) of actively growing mycelia from known isolates of the EU_6_A1 and EU_13_A2 genotypes (Table 1) and the unknown isolate were placed in the centre of the plate (pea medium) separated by 2–3 cm. The cultures were stored in the dark at 18°C for 18 days and formation of any oospores was checked under the microscope. If the known isolate was A1 and oospores were present, the mating type was determined to be A2, and conversely if the known isolate was A2 and oospores were formed, then the unknown isolate was determined to be A1.

2.4 | Metalaxyl-M sensitivity test

Six isolates from Pangalengan, Bandung (4), Parikesit, Wonosobo (1), and Wonokitri, Pasuruan (1) were used for metalaxyl-M sensitivity testing using a spiral gradient dilution method as described by Fairchild *et al.* (2013) and the poison agar method (Forbes, 1997). Pea agar medium was prepared as described above. In the spiral plating method, 50 ml pea agar was poured in each 100 mm Petri plate. After the medium was set, 10 g/L of metalaxyl-M was prepared from filtered Ridomil Gold EC (479.31 g metalaxyl-M/L; Syngenta Ag). Using a spiral plating robot, metalaxyl-M (54 μl) was placed in a spiral gradient on the plate so that the highest concentration of metalaxyl-M (10 g/L) was in the centre, with a gradient down to zero concentration at the edge of the plate. After 4 hr, lines were laid down on the medium from the outside to the inside of the plate with 10 μl of sporangia and zoosporangia (10^5 sporangia/ml). After 7 days, plates were scanned, and growth of mycelia was measured using Adobe Photoshop and the EC_{50} determined as described by Fairchild *et al.* (2013).

In the poison agar method, aqueous solutions of metalaxyl-M (prepared from Ridomil Gold EC as above) were added to flasks of pea agar (amended with RAN) to give final concentrations of either 0, 5, 10, or 100 mg/L, before 25 ml was poured into 50 mm plates. A plug of actively growing *P. infestans* mycelia from a 7-day-old culture was placed in the centre of the plate. After 7 and 10 days, radial growth of mycelia was measured, compared with the control culture plate, and sensitivity calculated as previously described by Forbes (1997).

2.5 | Multiplex simple sequence repeat genotyping

One-step multiplex simple sequence repeat (SSR) genotyping was performed using 12 microsatellite markers as described by Li *et al.* (2013). The Type-it microsatellite PCR kit was used for multiplex amplification (Qiagen). Twelve genotypes of *P. infestans* with known SSR alleles were also included in the PCR (Table 1). All PCR products from FTA cards were diluted at 1:50 (PCR product:PCR-grade water), and from genomic DNA were diluted at 1:100. PCR products were submitted to the Molecular Research Core Facility of Idaho State University (Pocatello, ID, USA) for fragment analysis. Fragment analysis was performed with an AB 3130xl Genetic Analyzer (Applied Biosystems). Each sample was processed in-line with a size standard GeneScan 500 LIZ (Life Technologies) for

FIGURE 1 (a) Map of Indonesia showing the three different regions, west, central and east Java. (b) Enlarged map of the island of Java showing the nine regencies of Java from where samples of *Phytophthora infestans* were collected (map developed in R)

fragment size determination. Raw data and sizing determination were analysed in GeneMapper software v. 5 (Applied Biosystems, Release 5.0, Build ID FC3, licensed by ISU Molecular Research Core Facility). Allele bins were set for each marker and alleles were scored.

2.6 | Data preparation and analysis

Microsatellite data was obtained from 146 samples. Ninety percent of samples were triploids ($n = 131$), with a few diploids ($n = 11$) and tetraploids ($n = 4$). To make the data uniform and polyploid, zero was added in missing alleles of diploid and triploid. Data from the same location from multiple years were combined and analysed based on geographical locations. Standard isolates (Table 1), data kindly provided by Dr David Cooke (The James Hutton Institute, Invergowrie, Dundee, UK), and some previously published data by Li *et al.* (2013) were compared for SSR fingerprints to assign specific genotypes. These data were also included in frequency-based analysis such as neighbour-joining (NJ) tree and minimum spanning network to see how our data correlated to them. Data were analysed in R package poppr v. 2.3.0. as described by Grünwald *et al.* (2017), Kamvar *et al.* (2014, 2015), and Shakya *et al.* (2018).

2.7 | Multilocus genotype analysis, diversity statistics, and mode of reproduction

Multilocus genotype (MLG), estimated multilocus genotypes (eMLG) after rarefaction, and diversity statistics for 146 polyploid data for 12 microsatellite loci were calculated using the R package poppr v. 2.3.0 (Kamvar *et al*., 2014, 2015; R Core Team, 2016; Grünwald *et al*., 2017). MLG diversity of a population was estimated using Shannon–Weiner Index in R package poppr (Shannon and Weaver, 1949; Kamvar *et al*., 2014, 2015). The expected heterozygosity (H_{exp}) was computed for all samples based on Nei's unbiased gene diversity (Nei, 1978). The mode of reproduction in each population was estimated based on index of association (I_A) and standardized index of association value ($\bar{r}_d$; Agapow and Burt, 2001; Kamvar *et al*., 2014, 2015). Both clone-corrected and non-clone-corrected data were used to calculate $\bar{r}_d$ and the significance was tested with 999 permutations in R package poppr (Kamvar *et al*., 2014, 2015). Allelic diversity was calculated based on Simpson's index and Nei's unbiased gene diversity in R package poppr (Simpson,1949).

2.8 | Population structure and differentiation

The genetic distance between the individual DNA samples was computed using a stepwise mutation model as described by Bruvo *et al*. (2004). A neighbour-joining tree was constructed based on Bruvo's distance with 1,000 bootstraps in poppr and the NJ tree was viewed and modified on Figtree v. 1.4.3 (Kamvar *et al*., 2014, 2015; http://tree.bio.ed.ac.uk/software/figtree/). NJ trees are useful to visualize how samples cluster and group together compared to the standard samples. Discriminant analysis of principal components (DAPC) was performed in R-package adegenet v. 2.0.1 to examine how the individual samples cluster to a population (Jombart, 2008; Jombart *et al*., 2010). Minimum spanning network (MSN) was constructed in poppr.

Pairwise fixation indices (F_{ST}) were calculated on clone-corrected data using Bruvo's genetic distance using R package strataG v. 1.0.5 (Archer *et al*., 2017). The F_{ST} values were used to examine the differentiation between the populations separated by geographical locations. Analysis of molecular variance (AMOVA) was performed based on Bruvo's genetic distance on clone-corrected data in R package ade4 v. 1.7-5 (Excoffier *et al*., 1992; Dray and Dufour, 2007). AMOVA estimates variance due to individual samples within the population or among populations.

3 | RESULTS

3.1 | Mating type and metalaxyl-M sensitivity test

Eighty percent of DNA samples were positively amplified with CAPS markers and determined to be the A1 mating type (Table 2). One sample (Indo 111) was identified as A2 mating type. However, the mating type could only be determined using CAPS and could not be confirmed using the Phyb markers. Six isolates were also tested for mating type using both the pairing test and CAPS markers and determined to be A1 mating type. Out of the six isolates tested for metalaxyl-M sensitivity, two isolates, Pasuruan and Wonosobo, were intermediate and four, from Bandung, were sensitive. See Table 2 for definition of categories used in the metalaxyl sensitivity test.

3.2 | SSR fingerprints

SSR fingerprints of our DNA samples were compared with standard data (our data, data provided by Dr David Cooke, and data published by Li *et al*., 2013) and the specific genotypes were assigned based on the same or close fingerprints. The specific genotypes were EU_2_A1 (60%), EU_4_A1, and EU_13_A2 (1.5%). The rest of the DNA samples were unique but closer to European genotypes than US genotypes. An NJ tree was constructed including standard isolates; our assigned genotypes clustered together with EU_2_A1, EU_4_A1, EU_13_A2, or separately (Figure 2). Mating type results also supported the assigned genotypes.

3.3 | MLG analysis, diversity statistics, and mode of reproduction

Genotypic diversity of individuals in a population was evaluated and a total of 131 MLGs were determined out of 146 DNA samples. The DNA samples from Wonosobo had a greater number of MLGs (36) and were more diverse compared to other regencies, followed by Pasuruan (24) and Bandung (23). DNA samples from Garut (6) and Majalengka (6) were the least diverse. DNA sample diversity was even in many regencies, with a value of 1.0 (Table 3). All of the regencies had a standardized index of association value greater than zero except Majalengka ($\bar{r}_d = -0.0235$, $p < .724$; Figure S1), supporting the evidence of clonal reproduction in eight regencies. An index of association with a zero value indicates sexual reproduction, whereas any value other than zero suggests asexual reproduction. Wonosobo, Pasuruan, and Bandung had a greater Shannon–Weiner index value compared to the other regencies, indicating a high diversity, and these regencies had a higher number of MLGs.

Allelic diversity of the 146 DNA samples based on geographic distribution was calculated using poppr. A total of 75 different alleles were detected from 146 DNA samples ranging from 2 (SSR2) to 21 (D13) alleles, with a mean of 6.25 alleles per locus (Table 4). Gene diversity was estimated using Simpson's index and ranged from 0.48 (SSR2) to 0.70 (D13), indicating that the SSR2 locus was the least diverse and D13 was the most diverse locus (Table 4; Figure S2). Nei's unbiased estimation was correlated with Simpson's index. Evenness values ranged from 0.42 (D13) to 0.97 (SSR6 and SSR8). The D13 locus had the highest number of alleles (21) and high allelic diversity with the least even distribution, resulting in large number of MLGs (Table 4; Figure S2). PiG11 (12) and Pi4B (8) also had a high number

TABLE 2 Phenotypic characterization of *Phytophthora infestans* isolates from nine regencies of Java, Indonesia

Isolates	Location	No. of DNA samples or isolates	Mating type	Mating type determination method	Metalaxyl-M sensitivity[a]
Indo147	Wonosobo	1	A1	Pairing test/PCR	Intermediate
Indo 148	Pasuruan	1	A1	Pairing test/PCR	Intermediate
Indo 143–146	Bandung	4	A1	Pairing test/PCR	Sensitive
Indo 1–28	Bandung	28 (NAmp = 5)	A1	PCR	NA[c]
Indo 29–34	Garut	6	A1	PCR	NA
Indo 35–40	Majalengka	6 (NAmp = 1)	A1	PCR	NA
Indo 41–50	West Bandung	10 (NAmp = 1)	A1	PCR	NA
Indo 51–69, 75–80, 131–140	Wonosobo	35 (Namp = 10)	A1	PCR	NA
Indo 70–74, 99–104, 118–130	Pasuruan	24 (NAmp = 5)	A1	PCR	NA
Indo 83–87, 111–117	Mojokerto	12 (NAmp = 1)	A1	PCR	NA
Indo 91–98, 141–142	Temanggung	10 (NAmp = 2)	A1	PCR	NA
Indo 81–82, 89–90, 106–110	Batu	9 (NAmp = 2)	A1	PCR	NA

Note: NAmp, not amplified in PCR and mating type not known; NA, not available as only DNA of the isolate was collected on FTA cards.
[a]Resistant: growth on both 5 and 100 mg/L poison agar plates with >40% growth compared to control (0 mg/L); intermediate: growth on 5 mg/L plate with >40% growth compared to control (0 mg/L); sensitive: <40% growth on both 5 and 100 mg/L plates compared to control (0 mg/L).

of alleles compared to the rest of the loci, with higher allelic diversity and less evenness, contributing to a larger number of MLGs.

3.4 | Population differentiation and structure

AMOVA was computed based on Bruvo's genetic distance on clone-corrected data to determine whether variation was due to individual DNA samples within a population or among populations (Table 5). Results showed that variation between populations (regencies) was only about 20% (Table 5). However, 80% of the variation was explained by individual DNA samples within the population, which was supported by the F_{ST} values. The F_{ST} calculated on clone-corrected data using Bruvo's genetic distance revealed low to modest differentiation between populations (Table 6), with values ranging from 0.02 to 0.12. The highest population differences were found between Garut and Majalengka (0.12), indicating that there is limited gene flow or limited migration of isolates between these regencies. Despite having the highest differentiation, these two regencies are geographically close. The lowest population differences were observed between Bandung and Wonosobo (0.02), even though these two regencies are not geographically close. Furthermore, the F_{ST} value indicates ongoing migration events between these two regencies. The least differentiation was between Wonosobo and the other regencies, even Majalengka, revealing two-way migration from Wonosobo, whilst the highest differentiation was between Majalengka and the other regencies (Table 6). The regencies of Wonosobo and Temanggung are geographically close and had modest differences between their populations (0.04). Likewise, Pasuruan, Mojokerto, and Batu are also geographically close, and had modest differentiation between their populations (0.07).

The discriminant analysis of principal components (DAPC) results support the small amount of variation among the regencies, because most of the populations (6 out of 9) were clustered together with the exception of populations from three regencies (Pasuruan, Bandung, and Batu; Figure 3). All 146 DNA samples from the nine regencies grouped together in four clusters. DNA samples from Bandung, Batu, and Pasuruan clustered separately, while the rest of the DNA samples clustered together to make the "mixed" fourth cluster. However, a few of the DNA samples from Wonosobo overlapped with other regencies, except Pasuruan (Figures 3 and 4). Most of our samples were also grouped together with standard data of EU_2_A1 (60%) EU_4_A1, and EU_13_A2 (1.5%), in an NJ tree based on Bruvo's distance (Figure 2). Standard US and other European genotypes clustered separately in the NJ tree.

4 | DISCUSSION

This study provides the first in-depth investigation into the genotypes that make up the *P. infestans* populations in the main potato-growing regions on the island of Java, Indonesia. Although in the past a few studies have been conducted with limited isolates, populations of *P. infestans* in Indonesia have not been thoroughly studied until now. Nishimura *et al.* (1999) included four samples obtained from an unknown location in Indonesia in 1993 while characterizing the *P. infestans* population in seven Asian countries and found that all four were A2 mating type and resistant to metalaxyl-M. In our study we only found one DNA sample, Indo 111, that was identified as A2 using CAPS markers. However, this mating type could not be confirmed using the Phyb markers. All of our DNA samples that were successfully amplified were determined to be the A1 mating type based on the CAPS marker results, and these results were

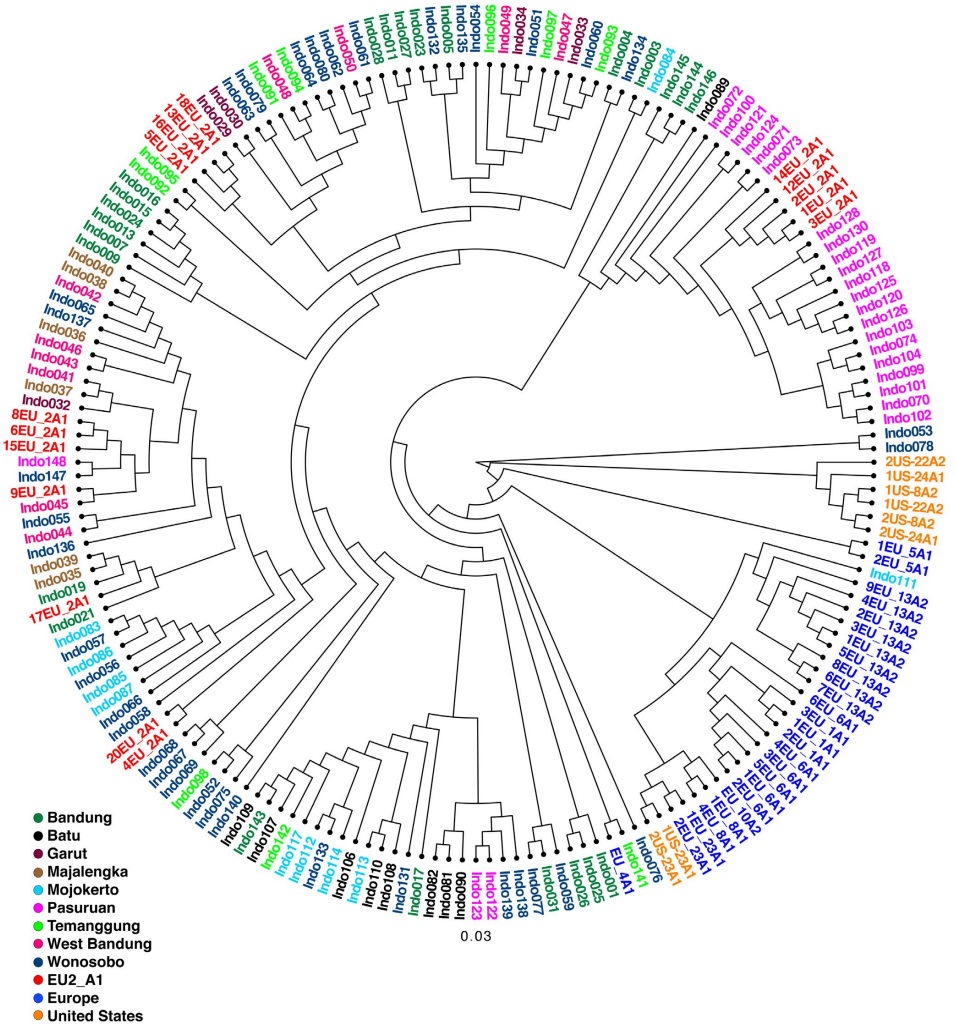

FIGURE 2 Neighbour-joining tree based on Bruvo's distance for *Phytophthora infestans* populations from nine regencies in Java, Indonesia, European and US standard isolates with 1,000 bootstrap replicates (data analysed in the R package poppr v. 2.3.0, results viewed and modified on Figtree v. 1.4.3). The colour of the isolate names indicates which location they are from

corroborated with the pairing test carried out with six isolates from Indonesia. Brylińska *et al.* (2018) have reported that isolates were not completely (100%) assigned to specific mating types using these molecular markers (CAPS and Phyb assigning isolates correctly by 96% and 84%, respectively). As such, Brylińska *et al.* (2018) recommended that local populations such as these need to be validated with the pairing test method before these markers are used for DNA samples.

Populations of *P. infestans* in Indonesia were reported to be resistant to metalaxyl-M (Nishimura *et al.*, 1999; Adiyoga, 2009). However, of the six isolates we tested for metalaxyl-M sensitivity, two were found to be intermediate and four were found to be

TABLE 3 Multilocus genotype and diversity statistics for microsatellite data for 12 microsatellite loci in populations of *Phytophthora infestans* from Indonesia by regency, collected from 2016 to 2019

Population	N [a]	MLG[b]	eMLG (*SE*) [c]	H [d]	H_{exp} [e]	Evenness	I_A [f]	$\bar{r}_d$ [g]
Bandung	32	23	9.18 (0.75)	3.08	0.597	0.943	0.781	0.0913
Batu	9	9	9.00 (0.00)	2.20	0.589	1.000	1.245	0.1989
Garut	6	6	6.00 (0.00)	1.79	0.587	1.000	1.261	0.1977
Majalengka	6	6	6.00 (0.00)	1.79	0.557	1.000	−0.111	−0.0235
Mojokerto	12	10	8.50 (0.58)	2.21	0.594	0.862	1.249	0.1479
Pasuruan	25	24	9.85 (0.00)	3.16	0.517	0.978	0.436	0.0596
Temanggung	10	10	10.00 (0.00)	2.30	0.565	1.000	1.917	0.2078
West Bandung	10	10	10.00 (0.00)	2.30	0.544	1.000	0.210	0.0451
Wonosobo	36	36	10.00 (0.00)	3.58	0.606	1.000	1.092	0.1162
Total	146	131	9.93 (0.26)	3.97	0.601	0.949	0.656	0.0662

[a]N, number of individuals per regency.
[b]MLG, number of multilocus genotypes.
[c]eMLG, expected number of MLGs for each regency.
[d]H, Shannon–Weiner index of MLG diversity.
[e]H_{exp}, Nei's unbiased gene diversity.
[f]I_A, index of association.
[g]$\bar{r}_d$, standardized index of association.

TABLE 4 Population statistics for clone-corrected microsatellite data for 12 microsatellite loci in populations of *Phytophthora infestans* from Indonesia

Locus	Allele	$1 - D$[a]	H_{exp} [b]	Evenness
D13	21	0.70	0.70	0.42
Pi4B	8	0.59	0.59	0.74
PiG11	12	0.61	0.61	0.62
Pi04	3	0.51	0.51	0.94
Pi63	5	0.66	0.66	0.89
Pi70	5	0.65	0.65	0.91
SSR2	2	0.48	0.48	0.95
SSR3	4	0.66	0.67	0.93
SSR4	6	0.66	0.67	0.83
SSR6	3	0.50	0.50	0.97
SSR8	3	0.65	0.65	0.97
SSR11	3	0.52	0.52	0.93
Mean	6.25	0.60	0.60	0.84

[a]$1 - D$, Simpson index.
[b]H_{exp}, Nei's, 1978 gene diversity.

TABLE 5 Analysis of molecular variance (AMOVA) for clone-corrected *Phytophthora infestans* populations based on Bruvo's genetic distance

Source	*df*	SS	MSS	% variance
Between regencies	8	3.095	0.387	19.870
Within regencies	125	10.763	0.086	80.130
Total	133	13.858	0.104	100

sensitive to metalaxyl-M (see Table 2). With our small sample size, it is impossible to determine how widespread resistance to metalaxyl-M is on Java. However, due to the genetic diversity of *P. infestans* isolates from Java, it is probable that there are isolates with a range of metalaxyl-M sensitivities on the island, depending on the local use of metalaxyl-M.

Microsatellite analysis revealed that most of our samples were EU_2_A1 or subclonal variants of EU_2_A1 based on the SSR fingerprints compared with already published data, our standards, and data kindly provided by Dr David E. L. Cooke (Li *et al*., 2013; David Cooke, The James Hutton Institute, Invergowrie, Dundee, UK, personal communication). Dr Louise Cooke also reported that the limited number of samples obtained from West Java as part of an AsiaBlight study were all EU_2_A1 (Queen's University, Belfast, UK, personal communication). However, we found large subclonal variation in the Indonesian *P. infestans* population.

Based on the SSR fingerprints and NJ tree analysis we found that about 60% of the population were EU_2_A1 with large subclonal variation. There are many reasons behind the large variation within clonal lineages. One is polyploidization, which is an increase in the number of alleles in a locus (Li *et al*., 2017). In our analysis, there were DNA samples that were diploid, triploid, and tetraploid. Most of our DNA samples were triploid ($n = 131$). Out of 12 loci, six were diploid, five were triploid, and D13 had a few DNA samples that were tetraploid (Figure S3). In addition to that, some loci had a higher number of alleles with high genetic diversity and less evenness, such as D13 and PiG11. The number of MLGs we found was increased due to polyploidization in alleles in six loci. It has been reported that clonal lineages

TABLE 6 Pairwise F_{ST} values for clone-corrected *Phytophthora infestans* populations from nine regencies in Indonesia

	Bandung	West Bandung	Batu	Pasuruan	Mojokerto	Wonosobo	Garut	Majalengka	Temanggung
Bandung	–								
West Bandung	0.05	–							
Batu	0.06	0.09	–						
Pasuruan	0.05	0.07	0.07	–					
Mojokerto	0.05	0.08	0.07	0.07	–				
Wonosobo	0.02	0.04	0.05	0.04	0.05	–			
Garut	0.07	0.09	0.11	0.10	0.10	0.06	–		
Majalengka	0.07	0.09	0.11	0.09	0.10	0.06	0.12	–	
Temanggung	0.05	0.07	0.09	0.08	0.08	0.04	0.09	0.10	–

Note: The lower the value, the greater the similarity between two populations.

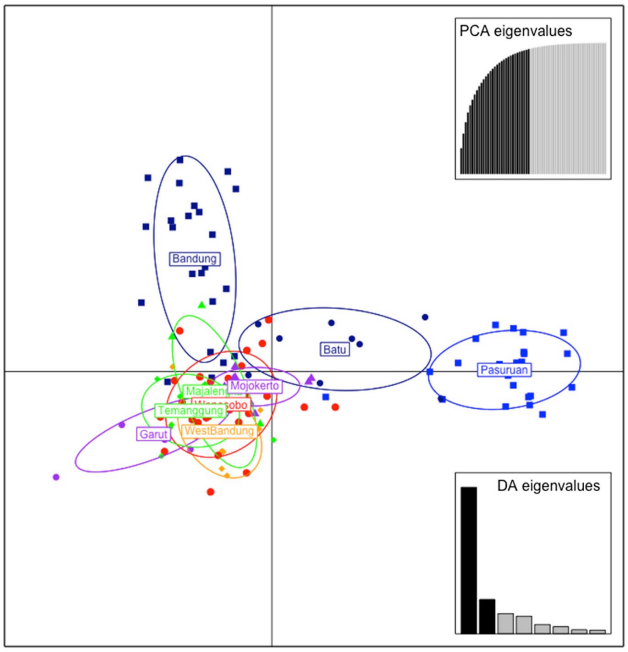

FIGURE 3 Discriminant analysis of principal components (DAPC) of *Phytophthora infestans* populations collected from 2016 to 2019 in nine regencies of Indonesia (data analysed in R package poppr v. 2.3.0)

tend to have more alleles (triploids) in multiple loci compared to progeny of sexual recombinants (Li *et al.*, 2013). The occurrence of polyploidization may mask accumulated deleterious alleles in asexual populations (Li *et al.*, 2017) that otherwise may lead to extinction during the course of evolution, according to Muller's law (Muller, 1964). That is why there is a prevalence of clonal lineages worldwide, as they are so successful due to their adaptability and fitness under many environmental conditions, even without sexual recombination (Li *et al.*, 2017). We also found clonal reproduction based on the linkage disequilibrium analysis (conducted with both raw and clone-corrected data) except in Majalengka. However, we had a very small number of samples from this regency and we need to include more samples to get a more precise result. Shakya *et al.* (2018) found a different trend; most of the *P. infestans* isolates in Mexico had diploid alleles in both sexual and clonal populations. However, Li *et al.* (2017) reported that most of the successful clonal lineages tend to be triploid because they have more heterozygous single nucleotide polymorphisms (SNPs) and a higher level of functional variations compared to diploids.

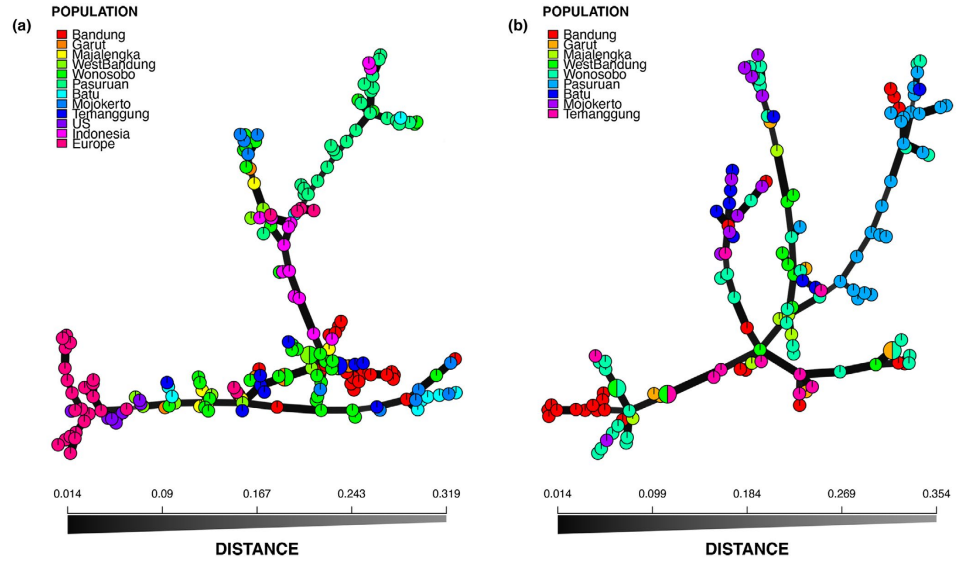

FIGURE 4 Minimum spanning network (MSN) of *Phytophthora infestans* isolates from Indonesia collected from 2016 to 2019 compared with some representative European and US isolates. (a) Samples from Indonesia and other standard samples from Indonesia, Europe, and the USA; (b) samples from Indonesia only

Another reason for subclonal variation may be mutation. Indonesia is right under the equatorial line and is subjected to high UV levels that may result in a higher mutation rate, as described in Ecuador (Delgado *et al*., 2013). Similar large subclonal variations of single clonal lineages were observed in Ecuador and India (Delgado *et al*., 2013; Dey *et al*., 2018). Large variations have also been reported in Nordic European countries (Brurberg *et al*., 2011). However, the variation in Nordic European countries is largely due to sexual recombination (Brurberg *et al*., 2011). Due to the subtropical climate, multiple potato crops can be grown throughout the year in Indonesia, India, and Ecuador so the disease cycle is maintained year-round. Li *et al*. (2017) stated that *P. infestans* isolates tend to decrease their number of alleles under adverse climatic conditions such as low carbon or exposure to sublethal levels of metalaxyl-M fungicide. In Indonesia, growers use fungicide sprays extensively, with up to 30 sprays in a single season (Adiyoga, 2009). This may have a negative impact on polyploidization (Li *et al*., 2017). However, factors such as high UV radiation, a continuous year-round disease cycle, small-scale growers who may not spray fungicides extensively, continuous use of susceptible cultivars, and very conducive environmental conditions for late blight in Java might lead to different scenarios where polyploidization levels are not impacted or are even increased. From our results we can speculate that the populations of *P. infestans* on the island of Java, Indonesia, are undergoing mutation through allele loss or gain.

The distribution of genetic variation in Javan populations was examined using AMOVA. Individual samples within the regencies contributed 80% of the variation compared to the populations between regencies that explained only 20% of the variation. We determined a similar trend in population differentiation. F_{ST} values, which ranged from 0.02 to 0.12, indicated that the differentiation between populations was fairly low to modest. In recent years, potato seed tubers have been locally produced in West Java, with limited seed imported from Germany and the Netherlands (Fugile *et al*., 2006; Adiyoga, 2009). The population differentiation analysis revealed evidence of higher migration between regencies of West Java and Central Java, such as between Wonosobo/Bandung, Wonosobo/West Bandung, and Wonosobo/Temanggung, than between West Java and East Java. However, we did observe similar levels of population differentiation between Wonosobo in Central Java and Pasuruan in East Java, suggesting there has been migration between populations in Central and East Java.

Genetic diversity was found to be highest in Garut, but in Majalengka it was found to be lower compared to other regencies. The discrepancy in genetic diversity from these regencies is most probably due to the limited number of samples collected, but could also be due to lack of migration or gene flow between these regions. The population differentiation based on F_{ST} values also supports low or lack of gene flow in these two regencies. Linkage disequilibrium analysis of

our DNA samples suggests that the mode of reproduction in *P. infestans* populations from Majalengka is sexual. However, out of six DNA samples, five were determined to be A1 mating type from this regency. So, the question arises as to how it can be possible to have sexual recombination without the A2 mating type? Because the number of DNA samples we collected from this region was so small it is most likely that there are A2 mating types in the region. Nishimura *et al.* (1999) reported the presence of A2 mating type isolates from Java so it is probable that there are A2 mating types in the population that were not collected in our samples. Majalengka was the only regency in Java with a population where we detected indications of sexual reproduction. More DNA samples and isolates need to be collected over multiple years to get a clearer view of the main mode of reproduction in this regency.

In the NJ tree based on Bruvo's genetic distance, we found that 62% of our DNA samples clustered with European isolates. However, 38% of DNA samples clustered separately from the European isolates, whilst one sample clustered with the US isolates. Some of the *P. infestans* populations in regencies such as Batu and Bandung are dominated by unique MLGs. The Batu population was composed of all unique MLGs. The Bandung population was mixed with a few EU_2_A1 (based on SSR fingerprints matched with published data) and unique MLGs, whereas the Wonosobo population was found to have a mixture of subclonal variants of EU_2_A1 and a few unique MLGs. The Pasuruan population was mainly dominated by EU_2_A1, which was also supported by DAPC analysis. We also found 1.5% of the samples recovered were EU_4_A1 or EU_13_A2. EU_2_A1 and EU_4_A1 are older European genotypes that were dominant in Europe over 20 years ago and EU_13_A2 was first identified in isolates obtained from the Netherlands in 2004. Martin *et al.* (2019) classified worldwide haplotypes of *P. infestans* and discovered these European genotypes all had the same haplotype (I-15 of Ia), and contributed this as maternal parents for other contemporary genotypes. Because Indonesia used to import Granola seed tubers from Europe, these genotypes may have been introduced to Java in infected symptomless seed tubers. The European genotypes most probably served as maternal parents on Java from which unique genotypes could have evolved. With the history of occurrence of the A2 mating type and resistance to metalaxyl-M, the presence of the EU_13_A2 clonal lineage in Mojekerto (in one DNA sample from the current study) and potential sexual reproduction in Majalengka, close monitoring and intensive sampling are necessary in these regencies.

This study provides insights into the structure and diversity of *P. infestans* populations on Java, Indonesia. In recent years, there have been many reports of late blight epidemics caused by older lineages of *P. infestans* and their increasing prevalence in different parts of the world (Njoroge *et al.*, 2019a). In east Africa, the US-1 clonal lineage seemingly dominated *P. infestans* populations since its introduction in Kenya in 1941 (Njoroge *et al.*, 2019a). However, it has recently been found that the US-1 lineage has been completely displaced in Kenya by EU_2_A1and almost completely displaced in other east African countries (Njoroge *et al.*, 2019a). The increased dominance of EU_2_A1 in east Africa has been attributed to its increased aggressiveness on potato (Njoroge *et al.*, 2019b). We discovered EU_2_A1 dominating *P. infestans* populations on Java, particularly in the East Javan regency of Pasuruan, which clustered separately from West and Central Javan regencies in the DAPC analysis. It is possible that EU_2_A1 is the original genotype that was introduced to Indonesia and it is now being displaced in Central and West Java by variants of EU_2_A1 and unique MLGs that we identified in these regions.

In Europe, programmes to monitor and track changes in populations of *P. infestans* over time have been used to enhance the effectiveness of current management strategies. These strategies have included host resistance management, fungicide programme optimization, the use of integrated pest management tools, and sophisticated decision support systems (Cooke *et al.*, 2011). In Indonesia, these systems do not exist and growers rely almost exclusively on the use of fungicides to produce viable yields of potatoes. With our studies showing that there are isolates of *P. infestans* with intermediate sensitivity to metalaxyl-M, a total reliance on fungicides for management of late blight is not the best option. Alternative sources of disease management such as resistant cultivars should be given consideration. One such alternative would be the development of bioengineered local varieties with 3 *R*-gene resistance to late blight such as the ones being developed by the USAID Feed the Future Biotechnology Potato Partnership (FtFBPP). This would be of significant benefit to potato growers in Indonesia because it would reduce the cost of production through less fungicide costs, reduce farmer exposure to fungicides, and reduce fungicide residues in food, land, and wastewater.

Before and after release of resistant cultivars in late blight-prone areas, it is very important to track the diversity of *P. infestans* genotypes over time, monitoring for factors or new isolates that may overcome resistance. This is critical to ensure the effectiveness and durability of any 3 *R*-gene cultivars that are released in Indonesia. The data from this study will serve as a baseline to inform development of integrated strategies to extend the efficacy and durability of the USAID FtFBPP 3 *R*-gene potato cultivars that are being developed for release in Indonesia.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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