DETERMINING EFFECTS OF MANAGEMENT PRACTICES ON POTATO EARLY DYING AND SOIL MICROBIOME AND ASSESSING RISK OF FUNGICIDE RESISTANCE IN *VERTICILLIUM DAHLIAE*

By

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An Abstract of the Thesis Presented
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Potato early dying (PED) is a yield-constraining soilborne disease of potato, caused by *Verticillium* spp. with *V. dahliae* being the predominant causal agent. Since the pathogen inhabits soil for long periods, PED management aims to reduce the population of *V. dahliae* in soil. Benzovindiflupyr and azoxystrobin are effective chemicals and frequently used in the control of *V. dahliae*. In this study, field trials were conducted at Aroostook Farm, Presque Isle, ME in 2019 and 2020. Chemical and biological products have been studied for PED control, and fungicide resistance was also examined. To evaluate fungicide resistance, benzovindiflupyr was characterized on sensitivity baseline and resistance risk development in *V. dahliae*. Benzovindiflupyr-resistant mutants of *V. dahliae* were generated, and evaluated for resistance stability, fitness, and pathogenicity. Results showed that most mutants maintained a high level of resistance and the same fitness and pathogenicity compared to their parents, indicating a high risk of resistance in fields. Therefore, the resistance of *V. dahliae* to benzovindiflupyr should be monitored in disease management. In field trials, Elatus (a.i. azoxystrobin and benzovindiflupyr), Aprovia (benzovindiflupyr), Stargus (*Bacillus amyloliquefaciens*) and Regalia (*Reynoutria sachalinensis* extract) were examined. In a second field trial, Vapam (a.i. metam sodium) was studied at three rates for soil

fumigation. Disease was evaluated during the growing season and postharvest. Bulked soil was sampled at different time points of the season. Soil DNA was extracted from the soil and root samples. Quantity of *V. dahliae* in soil was measured using quantitative polymerase chain reaction (qPCR). Soil microbial communities of soil from plots applied with Elatus at 280.9 ml/A and Vapam at 35 gal/A and 50 gal/A were examined using Illumina sequencing targeting the V4 region of the 16S rRNA gene for bacteria and ITS1 region for fungi. Results showed that all the fungicides and the fumigant significantly reduced PED disease incidences and *V. dahliae* population. Soil microbial community richness, abundance, and diversity were affected after Elatus and Vapam applications, and most bacterial and fungal families that recovered rapidly were non-pathogenic. All products can be used for PED control, but the impact on soil microbiome needs to be addressed.

DEDICATION

This would be a life-changing three years for me. I have gained way more than my expectation in my studies, hobbies, and love. I would be eternally grateful to my parents Hai Li and Ying Li for their encouragement and support in making this happen. During this period, I was most fortunate to meet my girlfriend, Shiyue Zhao, who lit up my life in the dark during this pandemic of COVID. She is the one who supports me in my life and studies, who makes me become a better me, and who makes me find and believe in my future path. Of course, another fortune was my car, BMW 650i, a car that once only appeared in my dreams. In the two and a half years I've been with her, she has never left me on the road. The scenery she took me to see, the road she took me on, are all wonderful experiences worth a lifetime of reminiscing.

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Chapter Abstract

The potato is a highly nutritious and valued food crop which has been constrained by many diseases, such as potato early dying (PED). PED is caused by *Verticillium* spp. with *V. dahliae* being the predominant causal species, which have a wide host range. It infects potatoes through the roots and blocks water and nutrient transportation in the vascular system, resulting in up to a 50% yield loss of potato. Therefore, PED control is dependent on reducing pathogen populations in the soil and utilizing plant resistance. Soil fumigation has been considered the most effective way to manage PED. Some biological control agents have potential for disease control, either through directly inhibiting the pathogen or by inducing plant resistance. Currently, synthetic fungicides are still a major and usually effective strategy. For example, azoxystrobin, a quinone outside inhibitors (QoIs) fungicide, and benzovindiflupyr, a succinate dehydrogenase inhibitors (SDHIs) fungicide, are frequently used. The downside of using chemical fungicides is fungicide resistance developed in pathogen populations. A risk assessment of fungicide resistance can be done by examining the sensitivity of numerous pathogen isolates to a specific fungicide, selecting resistant mutants, and testing the resistance and fitness of the mutants. All the above strategies affect soil microbiome, which is an important indicator of soil health, and can be analyzed using metagenomic tools to examine the abundance and diversity of the soil microbiome. Overall, PED should be managed in an integrated way and risk assessment should be implemented as part of the management operations.

1.1. The potato

Potatoes (*Solanum tuberosum* L.) were first discovered in the Andes mountains of South America and cultivated by indigenous people (Smith, 2012). They are within the genus *Solanum*, family *Solanaceae*, and order Solanales (KewScience, 2020). Potatoes are the third most important food crop for direct human consumption after rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.), and are the most important vegetable crop worldwide, as potatoes provide food security for millions of people. (CIP, 2017). The United States produced about 22 million tons of potatoes in 2017, making it the fifth largest potato producing country in the world (NPC, 2018). The farm-gate value for sales was approximately $3.77 billion in 2017 (NASS, 2018). In the same year, Maine ranked the sixth largest potato harvested area and ninth largest potato production state in the U.S. (NPC, 2018).

Potatoes are annual, herbaceous, dicotyledonous plants (CIP, 2017). The growth of potatoes takes about 75 to 150 days depending on the climate and variety (Smith, 2012). The growth can be divided into five stages, including sprout development, plant establishment (vegetative growth), tuber initiation, tuber bulking, and maturation (Thornton, 2020). When a seed potato is planted, it first breaks dormancy to start sprouting and grows to about 8 to 10 inches tall (Davies & Ross, 1984). Seed-borne diseases spread during this period since the nutrition is provided by the seed potato. After the leaves and stolons are grown, the plants enter a rapid growth phase until tuber initiation (Thornton, 2020). At this stage, the plants are threatened by soilborne or foliar diseases. Tubers are produced by transferring composite-leaves-produced starch to the underground stolon (CIP, 2017). Post-harvest diseases cause damage to tubers at this stage. Potato tubers can be used for consumption and as seed. Each year, 5% to 15% of the harvested potatoes are saved as seed potatoes for the next year (CIP, 2017). A mature tuber has 2 to 10 eyes. After a dormant period, eyes will germinate and grow into a new plant.

1.2. Potato diseases

Diseases are a major constraint to potato production. There are around 100 known diseases that can cause the loss of potato production to different extents (Hooker, 1981). Most diseases can be infectious (caused by biological pathogens) and the rest are non-infectious (caused by adverse environmental conditions) for potatoes (Agrios, 2005; Hooker, 1981). Potato plants can be affected by diseases at any part of the plant, such as roots, stems and shoots, either systemically or locally. Important infectious diseases include early blight (*Alternaria solani*), late blight (*Phytophthora infestans*), soft rot and blackleg (*Dickeya* spp. and *Pectobacterium* spp.), common scab (*Streptomyces* spp.), powdery scab (*Spongospora subterranea* f. sp. *subterranea*), potato mosaic virus (*Potato Virus Y*), potato mop top (*Potato mop-top virus*), root-knot nematode (*Meloidogyne* spp.), pythium leak (*Pythium ultimum* Trow var. *ultimum*), dry rot (*Fusarium* spp.), pink rot (*Phytophthora erythroseptica*), stem canker and black scurf (*Rhizoctonia solani*), and potato early dying (*Verticillium* spp.). Non-infectious diseases can be caused by plant genetic factors or congenital defects, unfavorable environmental factors, deterioration of chemical factors, excessive or insufficient supply of fertilizer elements, pollution, and phytotoxicities (Yang, 2018).

Plant diseases are not just the result of pathogen infection, but a consequence of the interactions among pathogen, host and environment (Scholthof, 2007). Both plants and pathogens require favorable environments for their growth, including but not limited to temperature, humidity, and pH conditions. The host plant passively provides adequate nutrition and space for the pathogens to multiply. Pathogen spreads by environment factors, such as rain and wind (Bauske, Mitchell et al., 2018; Ristaino et al., 2018). At the end of plant growth, the pathogen survives by a variety of methods into a next season to start a new round of infection.

1.3. Potato early dying (PED)

Verticillium spp. is a notorious pathogen of potato and causes potato early dying (PED), which is exacerbated by a lesion nematode, *Pratylenchus penetrans* (Martin et al., 1982). This disease makes infected potato plants mature 2 to 3 weeks earlier compared to normal growth conditions, and hence the yield is impacted negatively due to the tuber incomplete growth (CropIPM, 2009). Yield losses to PED typically range from 10% to 15% but can reach up to 50% in severely affected fields (K. B. Johnson et al., 1986; Powelson & Rowe, 1993). Since *V. dahliae* is predominant in most potato growing areas, it will be used as a representative species for discussion in this review.

1.3.1. Taxonomy and biology of *Verticillium dahliae*

The *Verticillium* genus comprises a small group of soilborne pathogens (Pegg & Brady, 2002). Historically, it was placed in class Deuteromycetes (Fungi Imperfecti) because its sexual stage had not been identified (Berlanger & Powelson, 2005). With the repeal of the Deuteromycota, *Verticillium* is considered as an anamorphic form of the *Plectosphaerellaceae* family in the Sordariomycetes class of the Ascomycota phylum (Inderbitzin et al., 2011). Currently, there are 10 species in the genus *Verticillium,* and seven of them are pathogenic to potato, including *V. albo-atrum*, *V. isaacii*, *V. nonalfalfae*, *V. nubilum*, *V. tricorpus*, *V. zaregamsianum*, and *V. dahliae* (Inderbitzin & Subbarao, 2014).

The hyphae of *V. dahliae* are hyaline, septate, multinucleate, and haploid (Berlanger & Powelson, 2005). Some hyphae specialize into branched conidiophores (Yu et al., 2016), the branching starts at the central axis, expands in a form of spokes (St-Germain & Summerbell, 1996). The name *Verticillium* was given by the verticillated structure of conidiophore (Berlanger & Powelson, 2005). Since the sexual stage has not been observed, it is described by ovoid- or ellipsoid-shaped conidia, which are borne on phialides—a structure borne by conidiophore (Gómez-Alpízar, 2001). Abundant black microsclerotia is also a feature of *V. dahliae*.

Microsclerotia are masses of melanized hyphae formed in host tissues at later stages of infection, which overwinter and stay viable in soil for over 14 years (Wilhelm, 1955; Slattery, 1981). *Verticillium dahliae* reproduces by production of massive numbers of conidia or germinates directly from microsclerotia.

1.3.2. Disease cycle of PED

After entering host plants, *V. dahliae* colonizes in the cortex of the roots as the roots absorb nutrients from the soil, and then moves through the xylem, forming conidia (Steere et al., 2015). Lesion nematodes can accelerate *V. dahliae* colonization by causing wounds from feeding on the roots to form a port of entry (Martin et al., 1982). Colonized *V. dahliae* reproduces by hijacking the upward transportation of water and minerals from the roots (Yadeta & Thomma, 2013). Hence, the symptoms of PED includes chlorosis and wilting, usually starting from the basal leaves after flowering (CropIPM, 2009). At the early stage of infection, the flow water and minerals are partially blocked due to the presence of *V. dahliae* in the xylem. Early symptoms develop in the lower portion of the plant and move to the top of the plant or to the newly developed shoots, resulting in the stem vascular system turning brown. In the later stages, chlorotic leaves transition to complete necrosis due to increased colonization within the plant (Berlanger & Powelson, 2005). The infected or dead plants in the field usually stand up higher than other plants, as a flag, and thus, called flagging (CropIPM, 2009). Meanwhile, tuber initiation also requires water and nutrients from roots. The symptoms shown on tubers include brown vascular discoloration on the stem end area (Steere et al., 2015). After host tissues senesce, microsclerotia are formed and remain in plant tissues for overwintering (Steere et al., 2015). *Verticillium dahliae* becomes saprophytic at this stage. When new hosts are planted and the environment is favorable, microsclerotia are induced by exudates from plant roots to germinate and conidia growth is promoted by xylem sap (Fradin & Thomma, 2006; Mace, 2012; Mol, 1995; Steere et al., 2015). Initiating a new round of infection.

1.3.3. Disease epidemiology

Verticillium dahliae cannot travel long distances by itself. However, human activities spread it by transporting infected potatoes. An example is that *V. dahliae* and *V. albo-atrum* were detected in 39% of seed potatoes during transportation in 1969 (Berlanger & Powelson, 2005; Easton et al., 1972). Once *V. dahliae* is carried to a field, microsclerotia can easily be spread by crop cultivation, rainwater, and irrigation water (Berlanger & Powelson, 2005). Therefore, *V. dahliae* can colonize the field as susceptible host plants are planted. Beyond that, inoculum densities and disease severity tend to increase year to year due to the high survival rate of microsclerotia, which allows them to spread when infected hosts die (Berlanger & Powelson, 2005; D. A. Johnson & Dung, 2010). *Verticillium dahliae* mainly remains in the top 30 cm from the soil surface and the density decreases as depth increasing, low numbers could still be found at greater depth (41 cm) of survival (Berlanger & Powelson, 2005). Yield loss of potato is often directly correlated with the amount of the pathogen in the soil (J. Li et al., 2017). A threshold of 5 to 30 cfu/cm^3 is the level observed for causing economic losses (Powelson & Rowe, 1993). The most favorable temperature for *V. dahliae* is about 21 to 27°C, and disease severity tends to maximize at temperatures near the higher limit (D. A. Johnson & Dung, 2010). For this reason, potato production may be threatened more by *V. dahliae* in Maine due to global warming. In addition, *V. dahliae* has a wide host range, comprised of more than 200 species of plants (Berlanger & Powelson, 2005; Klosterman et al., 2009). This wide host range makes it difficult to manage PED using crop rotation.

1.4. Soil treatment on PED control

1.4.1. Soil fumigation

Soil fumigation has been utilized for five decades and during that time thiophanate-methyl, chloropicrin, 1,3-dichloropropene, ethylene dibromide, 1,2-dibromo-3-chloropropane, methyl isothiocyanate (MITC), and ammonia gas are the most common products used (Gamliel

et al., 1997; Lembright, 1990). The mechanism of fumigation is to suffocate organisms, particularly pathogens, pests, and weeds by toxic gas released by the fumigant in the target area. Fumigation lowers the number of pathogens in the soil and often results in increased crop yield (Giovanni Bubici et al., 2019; Gullino et al., 2002; Leah Tsror et al., 2000; Yellareddygari & Gudmestad, 2018). Metam sodium (sodium N-methyldithiocarbamate) is a widely used fumigant, which takes effect through breaking down into the compound MITC, which is highly toxic, after applied in the soil (Zheng et al., 2006). Metam sodium is the active ingredient of the commercial product Vapam (AMVAC, Commerce, CA, United States). Soilborne fungi, soil arthropods, and nematodes can be eliminated due to its high toxicity (Lam et al., 1993). In studies conducted by Tsror et al. (2005), the application of metam sodium increased potato yield of up to 32%.

1.4.2. Chemical fungicides

Fungicides refer to a group of antifungal chemicals that kill or inhibit fungi. The Fungicide Resistance Action Committee (FRAC) has classified all common fungicides into 55 classes based on the mode of action (MOA) (Fisher et al., 2018; FRAC, 2020). Among all groups of fungicides, the group 7 quinone outside inhibitors (QoIs) and group 11 succinate dehydrogenase inhibitors (SDHIs) have potential to be effective in the control of PED. Both are mitochondrial-function-targeted fungicides with advantages such as broad-spectrum activity and high efficiency (Fernández-Ortuño et al., 2010; Sierotzki & Scalliet, 2013).

1.4.2.1. Quinone outside inhibitors (QoIs)

The fungicidal activity of QoIs acts as an inhibitor to block the electron transfer between cytochrome *b* and cytochrome *c*1 on the outer quinol-oxidation (Qo) site of cytochrome *bc*1 enzyme complex (complex III) (Fernández-Ortuño et al., 2010). It suppresses ATP production, and causes fungal cells to die due to energy deficiency (Fernández-Ortuño et al., 2010). The cytochrome *bc*1 is an integral membrane protein complex which functions critically in

respiration. The catalytic core formed by cytochrome *b*, cytochrome *c*1, and Rieske iron-sulfur protein (Fernández-Ortuño et al., 2010). The sites for catalytic reaction include the quinol oxidation (Qo) site and the Quinone reduction (Qi) site (N. Fisher & Meunier, 2008). Different QoIs may bind with different sites on this complex. Because the mode of action of QoIs is to inhibit the production of energy, it is more likely to slow down the growth of pathogens rather than kill them. Thus, the application timing of QoIs is critical—prior to infection or early stage of infection application is most effective (Fernández-Ortuño et al., 2010).

Azoxystrobin is a QoI fungicide, which was developed by Syngenta (Greenboro, NC, United States) in 1992 (Balba, 2007) and became popular in 1996 due to its board-spectrum disease-control capability (Balba, 2007; Fernández-Ortuño et al., 2010). Azoxystrobin is effective on many fungal pathogens such as *Alternaria alternata* (Bertelsen et al., 2001), *Plasmopara viticola* (Godwin & Cortesi, 1999), and *V. dahliae* (Syngenta, 2020).

1.4.2.2. Succinite dehydrogenase inhibitors (SDHIs)

The market adapted to newly produced SDHIs incredibly fast as many pathogens have been found to resist against the demethylation inhibitors (DMIs) and QoIs (Sierotzki & Scalliet, 2013). SDHIs function by affecting the respiratory electron transport system of pathogens by inhibiting the electron transfer in succinate dehydrogenase (SDH) complex (complex II) (Avenot & Michailides, 2010). The SDH complex plays a critical role in both the respiration system and tricarboxylic acid cycle. When succinate is oxidated to fumarate, the quinone (UQ) is reduced to ubiquinol (UQH_2) through the direct transition of the succinate-derived electrons without soluble NAD+ intermediates (Horsefield et al., 2006; Sierotzki & Scalliet, 2013). The SDH is also known as succinate ubiquinone reductase (SQR) that is composed of a membrane-peripheral domain and a membrane-anchor domain (Avenot & Michailides, 2010). The membrane-peripheral domain is the soluble part of the complex which oxidizes succinate to fumarate, it consists of two highly-conserved subunits—flavoprotein (Fp, *SDHA*) and iron-sulfur protein (Ip,

SDHB) (Avenot & Michailides, 2010). The *SDHA* contains a covalently FAD cofactor, while the *SDHB* contains three iron-sulfur clusters –[2Fe-2S], [4Fe-4S] and [3Fe-4S] (Avenot & Michailides, 2010; Ōmura & Shiomi, 2007). The membrane-anchor domain consists of two highly variable subunits—large subunits of cytochrome *b* (cybL, *SDHC*) and small subunits of cytochrome *b* (cybS, *SDHD*), contains specific binding sites for ubiquinone reduction and inhibitors, and anchors the catalytic subunits (*SDHA* and *SDHB*) to the inner mitochondrial membrane, thus facilitating the transfer of electrons to ubiquinone (Ackrell et al., 1992; Avenot & Michailides, 2010; Ōmura & Shiomi, 2007). In addition, there is a prosthetic heme b complexed between *SDHC* and *SDHD* (Ackrell et al., 1992; Avenot & Michailides, 2010). All those *SDHB*, *SDHC*, *SDHD*, and heme b group compose a ubiquinone binding pocket, and SDHIs specifically block the electron transport from the [3Fe–4S] cluster to ubiquinone binding pocket, hence the pathogens die from energy deficiency (Horsefield et al., 2006).

Benzovindiflupyr is a new SDHI fungicide, which was introduced by Syngenta in 2013 and commercialized in 2015 (EFSA, 2015; Wiglesworth & Tally, 2015). It is effective on pathogens such as *Colletotrichum* spp. (Ishii et al., 2016), *Sclerotinia sclerotiorum* (X. Huang et al., 2019), *Puccinia* spp., *Pseudomonas* spp., *Venturia inaequalis*, and *Verticillium* spp. (Syngenta, 2020).

1.4.3. Biological fungicides

Biologically based fungicides, or bio-fungicides can also suppress or kill plant pathogens. The major mode of actions of biological fungicides includes induced resistance in host, plant growth promotion, and antibiosis (Dicklow & Madeiras, 2018). Induced resistance has been mainly studied in Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR), which are mediated by pathogens and rhizosphere microbes, respectively (Romera et al., 2019). By applying resistance-inducing agents， plant diseases can be reduced by 20% to 85% (Walters et al., 2013). ISR is triggered by biological or chemical inducers and has a broad-spectrum

protection (Pieterse et al., 2014). Flavonoid compounds are chemical inducer of ISR, and extract of *Reynoutria sachalinensis* is one of many compounds that can trigger its accumulation (Osborne et al., 2009). Moreover, plant extracts such as canola, rapeseed, seaweed, and Canada milkvetch have been shown to effectively control *V. dahliae* (Uppal et al., 2008). Regalia (a.i. *Reynoutria sachalinensis* extract, Marrone Bio-Innovations, Davis, CA) is therefore being utilized as a potential resource for PED control. Regalia was introduced by Marrone and it is effective in controlling pathogens such as *Erysiphe* spp., *Alternaria solani*, *Fusarium* spp., *Rhizoctonia* spp., etc. by igniting the systemic resistance in plants (Marrone, 2020a).

On the other hand, plant growth promotion and antibiosis are also effective on *V. dahliae* control. Plant growth promoting bacteria (PGPB) can promote plant growth in various ways, such as biological nitrogen fixation, phosphate solubilization, phytohormone production, etc (Souza et al., 2015). Root-colonizing bacteria and fungi can suppress the pathogen with antimicrobial substances, or through parasitism or competition (Dicklow & Madeiras, 2018). In the management of PED, the available agents include *Bacillus* spp., *Pseudomonas* spp., *Leptosphaeria* sp., *Acremonium* sp., *Talaromyces* sp., etc. (Deketelaere et al., 2017; Y. Yuan et al., 2017), with *Bacillus* spp. being the most reported (Gomaa, 2012; Khan et al., 2018; B. Li et al., 2013; S. Li et al., 2013; Zhao et al., 2021). For example, *Bacillus subtilis* HJ5 can reduce infection of *V. dahliae* on cotton through root colonization and biofilm formation (S. Li et al., 2013); *B. licheniformis* and *B. thuringiensis* can suppress *Verticillium* spp. on soybean (Gomaa, 2012); and *B. amyloliquefaciens* has a prominent effect on controlling *V. dahliae* in vitro (B. Li et al., 2013). The commercial product Stargus (a.i. *Bacillus amyloliquefaciens* strain F727, Marrone Bio-Innovations, Davis, CA) has therefore become another candidate for PED control. It was also introduced by Marrone, and has been shown to effectively reduce the severity of diseases caused by *Sclerospora* spp., *Fusarium* spp., and *Botrytis cinerea* (Marrone, 2020b).

1.4.4. Other products for soil treatments

Soil organic amendments can be used in managing PED. For example, blood meal and fish meal were found decades ago to reduce the infection of *V. dahliae* on tomatoes. (Wilhelm, 1951). The mechanism of this activity was through high concentrations of volatile fatty acids under acidic soil conditions that reduce *V. dahliae* inoculum by breaking down into toxic substances (Conn & Lazarovits, 2000). Then, researchers found that many different kinds of animal or plant organic debris have been used to reduce the severity of disease caused by *V. dahliae* (Goicoechea, 2009; Inderbitzin et al., 2018; Tubeileh & Stephenson, 2020)

Crop rotation can help to reduce the severity of PED (LaMondia, 2006; Wheeler & Johnson, 2016), but studies have concluded that rotation with economically valuable crops is less effective for pathogens like *V. dahliae* which can infect more than 200 different host species (Borza et al., 2018; Larkin et al., 2010; Lazarovits & Subbarao, 2010). Interestingly, rotation with green manures can be more effective, as rotation with broccoli or other Brassica crops as green manures have been shown to be effective in reducing the inoculum of *V. dahliae*. The mode of action is through biofumigation, in which compounds in the mustard oils (glucosinolates) break down to produce isothiocyanate fumigants that have a broad spectrum fungicidal effect (Berlanger & Powelson, 2005; Xiao et al., 1998). In addition, sudangrass (*Sorghum vulgare* var. *sudanense* 'Monarch') and corn (*Zea mays* 'Jubilee') have also been used for the control of *V .dahliae* (Davis et al., 1996). High-glucosinolate mustard blend and a sorghum-sudangrass hybrid green manures also reduced the severity of disease caused by *V. dahliae* in field trials (Larkin et al., 2011). Irrigation can also be effective for PED control, although the requirement for available soil moisture is relatively high at up to 90% (Berlanger & Powelson, 2005; Cabral et al., 2020).

1.5. Fungicide resistance in pathogen

1.5.1. Mechanisms of fungicide resistance

Efficacy of some fungicides can be overcome by pathogen resistance through genetic mutation (Fernández-Ortuño et al., 2010). Pathogen resistance to a fungicide is developed in three steps: emergence, selection and adjustment (Van Den Bosch et al., 2011). At the early stage of fungicide application, there are only sensitive fungal pathogens in the community, and then resistant pathogens are selected because fungi have highly plastic genomes and reproduce rapidly (Fisher et al., 2018; Zhan et al., 2014). The population of mutated fungi will then continue to increase due to their competitive advantage and will outnumber the sensitive fungi within the population. As a result, the resistant mutants become dominant in the population and the efficacy of the fungicide is weakened or may even become ineffective completely (Zhan et al., 2014). Therefore, the application strategy is critical to lower the resistance risk.

QoI fungicides have a high resistance risk, mainly because the mutation usually occurs on only one gene - cytochrome b. This could result in a relatively high probability of mutation and consequently a high level of resistance in the pathogen (Zhan et al., 2014). Moreover, there are other ways for pathogens to survive under QoIs, such as the alternative oxidase (AOX) pathway which can sustain cyanide-resistant respiration (Fernández-Ortuño et al., 2010). Due to its high risk, azoxystrobin should be used with other products having different mechanisms of action.

The resistance against SDHI fungicides is usually induced by the mutation of different subunits of the SDH complex, which leads to changes in the protein structure (Avenot et al., 2008, 2009; Sierotzki & Scalliet, 2013). The conservative subunits of the SDH complex displayed a higher probability of point mutation (Zhan et al., 2014). As the SDHB is a highly conserved subunit in the membrane-peripheral domain, the SDHC and SDHD are highly variable subunits, and the mutation points of amino acids are on conserved positions of the ubiquinone binding pocket (Avenot & Michailides, 2010). In addition, the mutation point on SDHB is

monotonous, which means that the mutation usually happens on the same group of amino acids even among different pathogens, while mutation points on SDHC and SDHD are relatively variable (Zhan et al., 2014). However, it has also been reported that no mutation on SDH-related subunits were detected in some SDHI-resistant pathogens, which means there may be other mechanisms of resistance (Miyamoto et al., 2010). Resistance caused by SDH-related subunit mutations is usually detected in a variety of regions such as H/Y (or H/L) at 257, 267, 272 or P/L (or P/F) at 225, etc., which depend on different species of pathogens (FRAC, 2020; Sierotzki & Scalliet, 2013). In all, the resistance can commonly develop in the pathogen, but the cross resistance may not exist between the newly developed SDHI fungicides from the same class due to multiple mutation sites (Zhan et al., 2014).

1.5.2. Fungicide resistance risk evaluation

The probability of resistance development and the potential resistance level varies depending on the mode of actions of fungicides and the pathogen itself. The risk of fungicide application and the risk of pathogen resistance can be generally classified as high, medium or low levels based on the number of reported resistance occurrences (Brent & Hollomon, 1998). For example, *Botrytis cinerea*, the pathogen that causes gray mold, is a high-resistance risk pathogen because it has been reported to be resistant against 15 different fungicides (Hahn, 2014). To assess the risk of resistance requires massive field trial data. However, laboratory or greenhouse experiments can simulate the occurrence of resistance in the field. Resistant mutants can be obtained by exposing the initially sensitive cultures to a fungicide-amended environment. The risk evaluation therefore is determined by establishing a baseline sensitivity, and analyzing fitness, pathogenicity and cross-resistance of fungicide-resistant mutants (Brent & Hollomon, 1998; Egüen et al., 2015; Miao et al., 2016). The establishment of a sensitivity baseline is needed to understand how susceptible the wild type strains are. The resistance test for mutants determines the frequency of mutation and the level of resistance. It can be assessed by measuring

conidial germination and mycelial growth on fungicide-amended agar plates (Wise et al., 2008; Yuan et al., 2006), in a microplate (Egüen et al., 2015), or using a spiral plater which provides a gradient of concentrations in a Petri dish (Förster et al., 2004; Torres-Londoño et al., 2016). An effective concentration at 50% inhibition (EC_{50}) value is then calculated and used to determine the sensitivity. Fitness analysis investigates whether the resistant mutant could survive and dominate in field production. Pathogenicity analysis shows the possibility of whether the mutant will be a threat to production. Cross-resistance refers to whether one resistant mutant may develop resistance to another fungicide with a different mode of action, which can be used as a guide to develop fungicide application strategies. All the laboratory data can help to reveal the interactions between fungicides and pathogens, and therefore determine the risk (Brent & Hollomon, 1998).

There are three methods to determine the EC_{50} described above, which have their own advantages and disadvantages. The advantage of using toxic plates is that it is easy to perform, but the disadvantages are determination of the concentration range requires pre-experiment (too high or too low will impact on the results) and low efficiency for the pathogens with slow growth rate (Förster et al., 2004). The conidial germination method is done by making a series of conidial suspensions with different concentration of fungicide. The resistant individuals can germinate while spores without resistance are killed. Thus, the EC_{50} can be determined by calculating the spore germination rate, with the advantage of accuracy. However, the disadvantages of such a method are inefficiency of counting conidia under the microscope (Förster et al., 2004) and not being able to be performed on pathogens that do not produce conidia, such as *Rhizoctonia solani*. In contrast, the spiral plate works by linear sprinkling of fungicides on a plate, so that the medium contains the highest amount of fungicide in the central part while the lowest (zero) on the edge. The EC_{50} can be determined by calculating the fungicide concentration at where growth is 50% of the edge colony. The advantages of using

spiral plate are that it is extremely efficient and has a higher range and resolution at high fungicide concentrations. While disadvantages are the machine is costly and has relatively low accuracy.

The mutations occurring in fungicide resistance can be detected using molecular methods. For example, the resistance-related gene(s) of QoIs is on cytochrome *b* while SDHIs are *SDHA*, *SDHB*, *SDHC*, and *SDHD*. To examine a potential mutation on a specific gene, the target gene is amplified by polymerase chain reaction (PCR), and the PCR product can be sequenced and analyzed. By comparing changes of nucleotides, mutations can be identified. Mutations generally occur in only one gene for QoIs, which could be one of the reasons for its high risk. For SDHIs, on the other hand, the number and site of mutations can have different effects on resistance (Avenot et al., 2008, 2009; Miles et al., 2014).

1.5.3. Prevention of resistance development

In addition to risk evaluation of fungicides, an ideal way to prevent the risk of resistance is to develop fungicides with new modes of action (Fisher et al., 2018). However, the most practical way is to avoid or slow down the development of resistance by using fungicides with different modes of action and reducing the frequency and the dosage of fungicides (Zhan et al., 2014). Azoxystrobin and benzovindiflupyr are both effective in controlling *V. dahliae* with different modes of action and are combined in the formulated commercialized product Elatus (Syngenta, Greenboro, NC, United States).

1.6. Soil microbial communities

1.6.1. Soil microbiome

Soil is a natural medium that contains a wide variety of microorganisms, including bacteria, fungi, and archaea. All microorganisms within a certain area of soil comprise the soil microbiome. The abundance and structure of soil microorganisms is closely associated with soilborne diseases (Larkin et al., 2011; Stark et al., 2007). To suppress soilborne disease of

potato, there are a large number of beneficial microorganisms, including *Bacillus* spp., *Streptomyces* spp., *Pseudomonas* spp. and *Rhizobium* spp. (Nihorimbere et al., 2011). Most of the beneficial microorganisms can inhibit the growth of pathogens by secreting anti-biotic metabolites or by parasitizing pathogens (Dicklow & Madeiras, 2018; Nihorimbere et al., 2011). Moreover, beneficial microorganisms can also break down soil organic matter and promote humus formation (Vossbrinck et al., 1979); symbiosis with plants to promote plant growth (Prasad et al., 2015); absorb, fix and release nutrients (Roy & Singh, 1994); and reduce heavy metal contamination (Boopathy, 2000). There are also neutral microorganisms, which can suppress pathogens by competing for limited space and resources against pathogens since pathogens need host plants for reproduction (Abdullah et al., 2017).

Fumigants and fungicides not only suffocate pathogens, but also poison natural or beneficial organisms due to the broad-spectrum nature of them. By changing the proportion of bacteria and fungi in the soil, soil health may increase and proliferation of soilborne diseases decrease (Zhou & Ding, 2007). For example, it was observed that the diversity of the soil microbiome and the dominant population was changed after metam sodium application, and the dominant taxa were primary contributors to biological activity and healthy soil (J. Li et al., 2017; Sederholm et al., 2018).

1.6.2. DNA sequencing-based analysis on microbial communities

The abundance and diversity of soil microbial communities have been used as an indicator of soil health. However, due to fumigation and fungicide applications that can kill microorganisms in a broad spectrum, reduced populations of beneficial microorganisms is a concern for plant health (Neilson et al., 2020; Podio et al., 2008).

The next-generation sequencing (NGS) technologies are a powerful tool for the analysis of soil microbial communities (Coats et al., 2014; van Elsas et al., 2008). NGS allows parallel sequencing of large-scale DNA amplicons, and makes possible for genotyping of hundreds to

thousands of samples in one run (Shendure & Ji, 2008). Five platforms in NGS are available: 454 pyrosequencing, Illumina/Solexa, SOLiD, Ion Torrent technology, and PacBio RS (Krishna et al., 2019; Liu et al., 2012). Illumina sequencing platform is chosen by more researchers as it is more efficient and cost effective than other platforms (Luo et al., 2012). Miseq is a next generation sequencing instrument of Illumina. It features reversible-terminator sequencing-by-synthesis technology to provide end-to-end sequencing solutions. Different amplicons can be used for different variable regions sequencing. For example, 16S ribosomal RNA (rRNA) v4 hypervariable region is used for bacterial community analysis while internal transcribed space1 (ITS1) hypervariable region is used for fungal community analysis (Huang et al., 2015). The data set generated by NGS is subjected to bioinformatic pipelines such as quantitative insights into microbial ecology (QIIME) and Mothur for analysis (López-García et al., 2018). The operating taxonomic units (OTUs) and amplicon sequence variants (ASV) can be used for classifying the sequences (Prodan et al., 2020).

Microbial community analysis is frequently performed using three estimators. Alpha diversity is for within-community analysis; beta diversity is for between-community analysis; and gamma diversity is for between-region diversity analysis (Sepkoski Jr, 1988). Therefore, soil microbial community richness and diversity can be analyzed by alpha diversity, and the data can be presented as statistical indices such as Chao, Ace, Shannon, and Simpson (J. Li et al., 2017). Chao is an index for estimating the number of OTUs contained in a sample using the chao1 algorithm. Chao1 is commonly used in ecology to estimate the total number of species (Chao, 1984). Ace is an index used to estimate the number of OTUs in a community (Chao et al., 2006). Shannon and Simpson indices are commonly used to reflect the alpha diversity (Shannon, 1948; Simpson, 1949). The higher the Shannon index, the higher the diversity, while the opposite of Simpson index.

1.7. Conclusions

PED is an important soilborne disease of potato that threatens potato production. Due to *V. dahliae* features long life span and wide host range, the control strategy is mainly based on the reduction of inoculum. Therefore, the most widely used control methods include soil fumigation and applying chemical fungicides. Soil fumigation and chemical fungicides not only affect pathogens, but also poison natural or beneficial organisms. It is of great importance to understand and evaluate the outcome of fumigant and chemical fungicide applications on the alteration of the soil microbiome, especially beneficial microorganisms. One of these alterations is pesticide resistance and risk evaluation.

CHAPTER 2

SENSITIVITY AND RESISTANCE OF *VERTICILLIUM DAHLIAE* TO BENZOVINDIFLUPYR

Chapter Abstract

Verticillium dahliae is a soilborne pathogen causing potato early dying resulting in significant losses to potatoes. Benzovindiflupyr, a succinate dehydrogenase inhibitor (SDHI), has been commonly used as an effective fungicide in controlling *V. dahliae*. However, frequent applications of a fungicide may result in the development of fungicide resistance in the pathogen. To evaluate the risk of benzovindiflupyr resistance, 38 *V. dahliae* isolates were obtained from potato stems showing early dying symptoms in Maine. Sensitivity was determined and a baseline was established based on the effective concentration for 50% inhibition (EC_{50}) values, which ranged from 0.07 to 11.28 µg/ml. By exposing *V. dahliae* to a high concentration of benzovindiflupyr, 18 resistant mutants were obtained from eight isolates, with EC_{50} ranging from 18 to more than 1000 times higher than their parental isolates. To examine their fitness, the mutants were continuously cultured up to the 10^{th} generation. Mycelial growth, conidial production, competitiveness, pathogenicity, and cross-resistance of the 10^{th} generation mutants were further examined. Results showed that 50% and 60% of the resistant mutants retained the adaptive level in mycelial growth and conidial production similar to their parents. All mutants had no changes in pathogenicity. No cross resistance was detected in the mutants between benzovindiflupyr and either azoxystrobin, boscalid, fluopyram, or pyrimethanil. Moreover, all mutants were highly sensitive to azoxystrobin. Thus, the resistance of *V. dahliae* to benzovindiflupyr should be considered in fungicide management, and azoxystrobin could be a candidate used along with benzovindiflupyr to reduce the risk of resistance.

2.1. Introduction

Verticillium dahliae is a notorious soilborne pathogen that features a long life-span and wide range of hosts. Microsclerotia serve as a survival structure, allowing *V. dahliae* to stay viable in soil for over 14 years (Wilhelm, 1955). *Verticillium dahliae* can infect more than 200 species of plants, including many economically important crops such as potatoes, cotton, strawberries, and alfalfa (Berlanger & Powelson, 2005; Klosterman et al., 2009). When the conditions become favorable, *V. dahliae* infiltrate into the cortex of potato roots and produce a massive number of conidia to colonize in the xylem (Martin et al., 1982). As a result, upward transportation of water and minerals from the roots is blocked (Yadeta & Thomma, 2013).

Currently, controlling *V. dahliae* relies on soil fumigation and chemical fungicides, although cultural practices are also important (Gamliel et al., 1997; Lembright, 1990; Syngenta, 2020). Among the fungicides available in the market, Elatus and Aprovia (both Syngenta, Greenboro, NC, United States) are most promising products. Benzovindiflupyr is the active ingredient in both fungicides (EFSA, 2015; Wiglesworth & Tally, 2015). Benzovindiflupyr functions as blocking the electron transport from the [3Fe–4S] cluster to ubiquinone binding pocket, resulting in the death of pathogens through energy deficiency (Horsefield et al., 2006). This mode of action is known as a succinite dehydrogenase inhibitor (SDHI).

As a respiratory inhibitor, the occurrence of pathogen resistance is inevitable. Although SDHI fungicides target multiple genes of pathogens, the risk is considered medium to high by the Fungicide Resistance Action Committee (FRAC, 2020). Therefore, naturally occurring resistance to benzovindiflupyr should be monitored. Risk assessment of fungicide resistance can be conducted through laboratory or greenhouse experiments, which mimic the occurrence of resistance in the field in a much more efficient way (Brent & Hollomon, 1998). By establishing a baseline sensitivity of natural populations of pathogen, any change of sensitivity can be noticed. Once a resistant strain occurs, its risk is determined by its adaptation, fitness, pathogenicity and

cross-resistance to other fungicides (Brent & Hollomon, 1998; Egüen et al., 2015; Miao et al., 2016). Resistant mutants can be obtained by exposing wild-type (initially sensitive) cultures to the fungicide. Fitness indicates whether the resistant mutant can survive and dominate in field production. Pathogenicity assay shows the possibility of whether the mutant will be as virulent and aggressive as the wild type. Cross-resistance assessment gives an indication of whether another fungicide is effective if resistance occurs.

Benzovindiflupyr resistance in *V. dahliae* has not yet been reported. The objectives of this study were to examine the natural population of *V. dahliae* in benzovindiflupyr sensitivity and to determine the risk of benzovindiflupyr resistance.

2.2. Materials and Methods

2.2.1. *Verticillium dahliae* isolates

Verticillium dahliae isolates were obtained from potato stems and tubers showing disease symptoms. Twenty-nine isolates were from two locations in Houlton, Maine: GPS 46.137691; -67.835751 and GPS 46.098363; -67.892044. The remaining 19 isolates were obtained from Hao Lab, University of Maine, Orono, Maine, most of which were isolated in previous years from various locations. Isolation was performed by cutting either the wilted stems with microsclerotia or discolored tubers into 0.5-cm-long pieces, disinfestation with 75% ethanol for 30 seconds followed by 10% bleach solution for 5 min, and then immediately rinsing with sterile tap water. The disinfested pieces were placed onto the semi-selective medium NP-10 in a Petri plate (Kabir et al., 2004). Pure cultures of each isolate were derived using a single spore method on potato dextrose agar (PDA) plates. Genomic DNA of *V. dahliae* isolates was extracted by FastDNA spin kit (MP Biomedicals, Solon, OH, United States), which was used as a template for performing polymerase chain reaction (PCR) targeting the ITS gene. PCR products were sequenced and analyzed using BLAST algorithm against the National Center for Biotechnology Information Search (NCBI) database for fungal identification.

2.2.2. Fungicides

Technical grade benzovindiflupyr (97% active ingredient) was provided by Syngenta Crop Protection Inc. (Greensboro, NC, United States). The stock solution was prepared by dissolving the fungicides into methanol to make a concentration of 10,000 µl/ml, which was stored at 4ºC in the dark. In addition, technical grade azoxystrobin (96% active ingredient, Syngenta, Greenboro, NC, United States), boscalid (98% active ingredient, BASF, Research Triangle Park, NC, United States), and commercial products Velum prime (a.i. 41.5% fluopyram, Bayer, Whippany, NJ, United States), and Luna Tranquility (a.i. 25% fluopyram and 75% pyrimethanil, Bayer, Whippany, NJ, United States) were also prepared for the cross-resistance study. Salicylhydroxamic acid (SHAM) (a.i. 99%, Sigma-Aldrich, Sigma-Aldrich, St. Louis, MO, United States) was used as an auxiliary inhibitor for alternative oxidase pathway.

2.2.3. Sensitivity of *Verticillium dahliae* to benzovindiflupyr in vitro

Conidia germination assay was used for resistant mutants and their parental isolates. A 6-mm-diameter culture plug of *V. dahliae* was transferred into a 2 ml vial containing 1 ml potato dextrose broth (PDB) and incubated at 22ºC for 5 days for conidial suspension. Twenty microliters of conidial suspension adjusted to 10^5 conidia/ml were added to 20 µl benzovindiflupyr at concentrations of either 10, 5, 2.5, 1.25, 0.625, 0.3125, or 0.15625 µg/ml on a well of a repression-welled glass slide. Sterile tap water was used as a negative control. The slide was incubated in a moist container. Each treatment was triplicated. After 15 to 24 hours of incubation, the number of germinated conidia was counted under a microscope when that of the negative control reach 80%. The effective concentration for 50% growth inhibition (EC_{50}) value was estimated for each isolate by regressing the probit-transformed relative germination inhibition on the logarithm of fungicide concentration.

In a second method, spiral plating method was employed (Förster et al., 2004). Benzovindiflupyr solution at 500 µg/ml was spirally distributed by an Autoplate 4000 plater onto

a 15-cm Petri dish containing 58 ml of PDA (Spiral Biotech, Inc., Norwood, MA, United States). The 100 µl exponential mode was used to make the final concentration range from 0.05 µg/ml to 108.17 µg/ml. Conidial suspension was prepared by transferring 3 plugs of 5-day old *V. dahliae* cultures into a flask with 10 ml of sterile tap water. Three autoclaved wood stirrers (5.2 cm x 0.6 cm) were soaked into the conidial suspension and shaken for hominization. After 10 min, those conidia-carried wood stirrers were placed across the fungicide-amended PDA plate. The plate was sealed with parafilm and incubated for two weeks at room temperature. The position of 50% growth of *V. dahliae* was recorded and the track number was then measured (Figure 2-1). Concentration of each radius was calculated as $[(V_{local} / V_{agar})/1000]$ x fungicide concentration x dilution factor, where V_{local} was the agar volume of each radius track, V_{agar} was total agar volume, and dilution factor was technical numbers provided by the manufacturer.

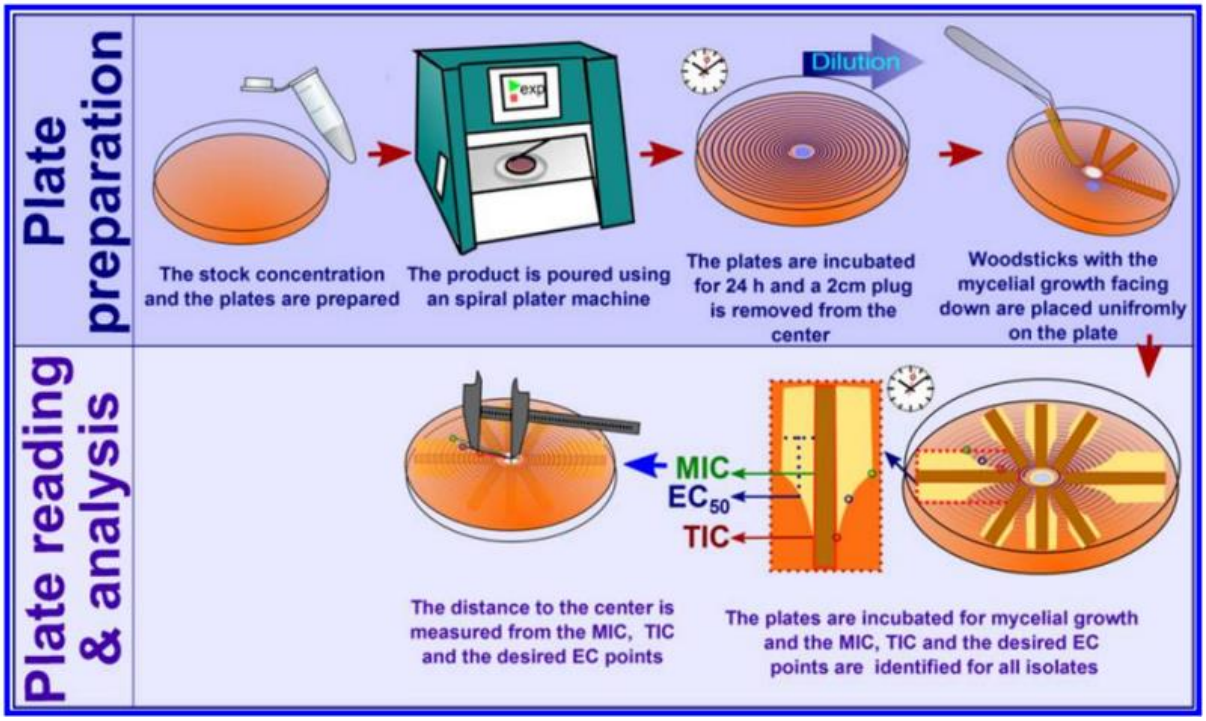

Figure 2-1. Schematic diagram of spiral plating procedure. Modified from Torres-Londoño et al., 2016.

2.2.4. Generation of benzovindiflupyr-resistant *Verticillium dahliae* mutants

Eight *V. dahliae* isolates were chosen for resistant mutant induction. One plug of a 5-day-old culture was transferred into a 2 ml vial containing 1 ml of 20 µg/ml benzovindiflupyr-amended PDB. The cultures in the liquid medium were incubated for three days in the dark. An aliquot of 100 µl conidial suspension was spread on a 20 µg/ml benzovindiflupyr-amended PDA plate, which was incubated for 2 days at 22ºC. Fungal growth on the plate was considered as a mutant and transferred to a new PDA plate for storage. The mutant was then subjected to a conidial germination test for sensitivity. Resistance factor (RF) was used to show the fold increase in resistance and calculated as EC_{50} of mutant / EC_{50} of parental isolate.

2.2.5. Resistance stability

Mutants were transferred to a fungicide-free PDA plate and incubated at 22ºC. After 2 days of incubation, the newly grown colony was transferred to another new PDA plate, which was repeated for 10 generations. EC_{50} was determined by the conidial germination method on the transfer of 10^{th} generation, and the RF was then calculated.

2.2.6. Fitness of *V. dahliae* mutants

2.2.6.1. Mycelial growth and conidial production

Ten 10^{th} generation mutants with 10 times or greater RF value were chosen for the following test. A 6-mm-diameter agar plug was cut from the cultures with a cork borer and placed on a PDA plate. After a 7-day incubation at 22°C, colony diameter was measured perpendicularly. Each culture was triplicated.

After the mycelial growth assay, a 6-mm-diameter agar plug of both mutants and their parental isolates were transferred into a 2 ml vial containing one milliliter of sterile tap water. Ten microliters of suspension was transferred to Bright-Line™ hemocytometer (25×16) (Sigma-Aldrich, St. Louis, MO, United States) for estimating conidial production. Each conidial suspension was measured three times. Number of conidia in the four corners and the central

square was recorded. Total number of conidia per milliliter was then calculated as (Sum of recorded conidia number / 80) x 400 x 10,000.

2.2.6.2. Competitiveness of *V. dahliae* mutants

Conidial suspensions of mutants (M) and wild-type isolates (W) were made by transferring one plug (d = 6 mm) of culture into one milliliter of sterile tap water. The suspensions of the M and the W were mixed in ratios of M:W = 0%:100%, 25%:75%, 50%:50%, 75%:25%, and 100%:0%. One hundred microliters of each mixed suspension were evenly spread onto a PDA plate. After three days of incubation, a 6-mm-diameter agar plug was cut and placed into a 2 ml vial containing one milliliter of PDB. Twenty microliters of conidial suspension were mixed with the same volume of 10 µg/ml benzovindiflupyr to make the final concentration of 5 µg/ml. Conidial germination was determined after 20 hours of incubation at 22°C in a 100% moisture chamber. Competitiveness was estimated by comparing the expected germination ratio to the observed germination ratio. The expected value was obtained by comparing the germination ratio of the wild-type isolate and mutant under benzovindiflupyr-amended condition, calculated as (Germination ratio $_{\text{mutant}}$ x M:W) + Germination ratio $_{\text{wild-type}}$ x (1 - M:W).

2.2.6.3. Pathogenicity of *V. dahliae* mutants

A greenhouse experiment was conducted in Roger Clapp Greenhouse, University of Maine, Orono, ME. Potato variety ‘Superior’ seed pieces were planted in on 1-gallon plastic pots containing potting soil. Plants were watered as needed after emergence. Six weeks after planting, conidial suspensions of mutants and their parental isolates were prepared by washing off conidia from a 7-day old culture on a 9-cm PDA plate. The suspensions were then diluted to a concentration of 10^6 conidia/ml (Dung et al., 2013). Two hundred milliliters of conidial suspensions were poured into the soil along plant stem. There were four replicates per treatment. Disease was evaluated three weeks post inoculation by cutting potato stems or stem-end tubers for the examination of vascular discoloration. Disease severity was categorized into four levels,

with 0 = healthy, 1 = discoloration in vascular system but no symptom on plant, 2 =chlorosis of potato plant or slight discoloration on tuber, 3 = wilting of potato plant or discoloration on tuber, 4 = dieback potato plant (Figure 2-2 and Figure 2-3).

Figure 2-2. Potato early dying plant symptom in greenhouse. A: healthy potato plant, B: chlorosis and wilting of potato plant, C: dieback of potato plant.

Figure 2-3. Potato early dying vascular symptoms on potato stem and tuber. A: healthy stem, B: discoloration of stem, C: healthy tuber, D: discolored stem-end tuber.

2.2.6.4. Cross-resistance

All test fungicides were prepared at a concentration of 500 µg/ml. Each fungicide was distributed on 15-cm PDA plates using the spiral plating method as described above. EC_{50} was determined on *V. dahliae* mutants and their parental isolate by culturing them on the fungicide-amended PDA plates. There were three replicates per treatment. For azoxystrobin test, SHAM

was evenly amended on the plate at the final concentration of 100 µg/ml to inhibit alternative oxidase pathway.

2.2.7. Statistical analyses

Data was analyzed using SPSS 27 software (IBM Corp., Armonk, NY, United States). A one-way analysis of variance (ANOVA) was used to compare differences of mycelial growth, conidial production, and disease severity. Chi-square goodness-of-fit test was used to compare the theoretical expected ratios of germination for competitiveness test. Spearman's rank correlation test was used for examining cross resistance between benzovindiflupyr and other tested fungicides.

2.3. Results

2.3.1. Baseline sensitivity of *Verticillium dahliae* to benzovindiflupyr

A total of 38 isolates were subjected to sensitivity test by using either spiral plating or conidial germination. The EC_{50} of tested isolates ranged from 0.07 to 11.28 µg/ml, where 37% of them were distributed between EC_{50} of 0.51 and 1.00 µg/ml, and 89% showed EC_{50} <3.0. A gap was observed between 3.51 and 4.50 µg/ml. Three relatively resistant isolates with EC_{50} greater than 5 µg/ml were found (Figure 2-4).

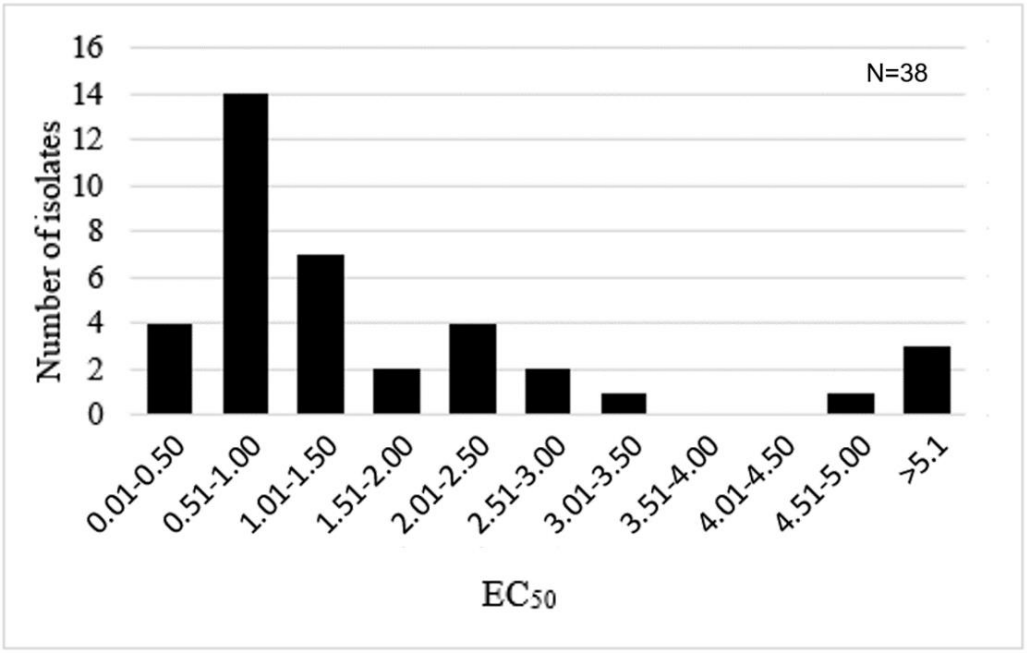

Figure 2-4. Distribution of effective concentration at 50% inhibition (EC_{50}) values (µg/ml) to benzovindiflupyr for 38 *Verticillium dahliae* isolates.

2.3.2. Mutants and their resistance stability

Eighteen mutants were obtained from eight *V. dahliae* isolates. RF values ranged from 4.2 to over 1000 (two with RF < 1 were excluded), while most of them were between 0 and 49, accounting for 39%, followed by 50 to 99 and >1000 (Figure 2-5). Ten mutants were chosen for resistance stability test. After 10 generations of continuous culture, 30% isolates showed reduced resistance, while the other 70% showed increased resistance (Table 2-1). The largest increase in EC_{50} was found in H7M4, with an increase of 236 µg/ml after 10 generations of culture. In addition, those 10^{th} generation mutants with reduced EC_{50} were still more resistant than their parental isolates.

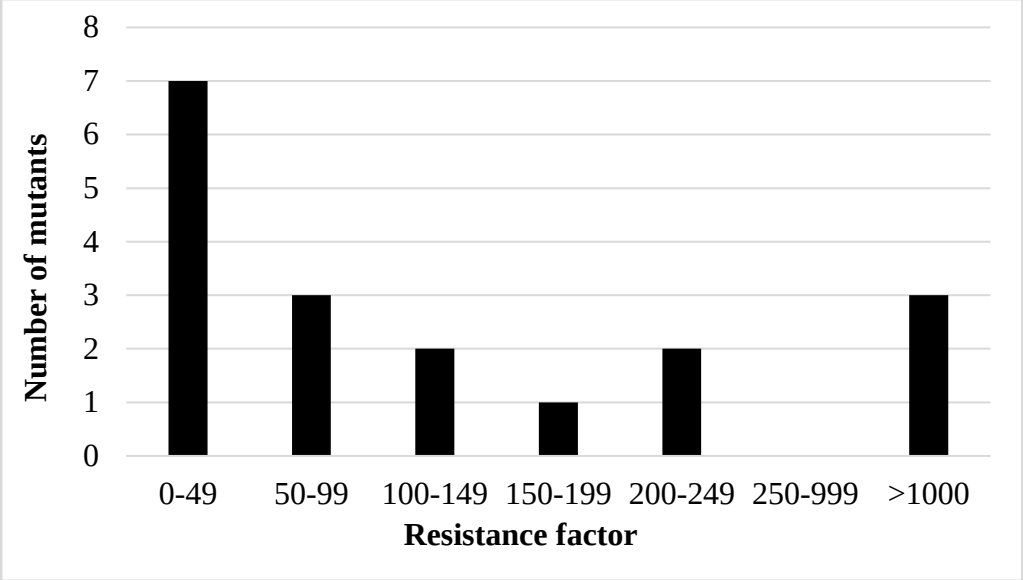

Figure 2-5. Distribution of resistance factor (RF) for 18 *Verticillium dahliae* resistant mutants to benzovindiflupyr. RF was calculated as EC_{50} of mutant / EC_{50} of parental isolates.

Table 2-1. Stability of benzovindiflupyr-resistant *Verticillium dahliae* mutants

Parental isolate	Mutant	EC_{50} (µg/ml) [z]			Difference [y]
		Parental isolate	1st generation mutant	10th generation mutant	
H1	**H1M2**	0.704	20.190	13.000	↓
H2	**H2M1**	0.940	34.580	43.870	↑
H2	**H2M3**	0.935	0.750	61.410	↑
H4	**H4M2**	0.623	31.800	14.40	↓
H5	**H5M3**	0.759	78.030	152.00	↑
H7	**H7M1**	0.113	14.750	7.590	↓
H7	**H7M2**	0.113	10.030	15.180	↑
H7	**H7M3**	0.113	8.930	35.000	↑
H7	**H7M4**	0.113	8.760	245.000	↑
H14	**H14M1**	2.061	0.003	152.000	↑

[z] EC_{50}: Effective concentration at 50% inhibition (EC_{50}). [y] Difference between 10th generation mutants and their 1st generation mutants. ↑: EC50 increased for 10th generation mutants, ↓: EC50 decreased for 10th generation mutants.

2.3.3. Fitness of *V. dahliae* mutants

2.3.3.1. Mycelial growth and conidial production

After one-week of incubation, 50% of the mutants showed different growth rates from their parental isolates ($P < 0.01$). Among them, three of the five had significantly slower mycelial growth rates than their parents, while the other two grew significantly faster (Table 2-2). However, a different pattern emerged in the conidia production test, where 60% of the mutants did not show a significant difference in conidia production compared to the parental isolates, and the other 40% produced less conidia than their parental isolates ($P < 0.01$) (Table 2-3).

Table 2-2. Mycelial growth (colony diameter) [z] of benzovindiflupyr-resistant *Verticillium dahliae* mutants and their parental isolates

Parental isolate	Mutant	Mutant growth (cm)	Parental isolate (wild-type) growth (cm)	*P*
H1	**H1M2**	1.78	2.23	0.002
H2	**H2M1**	2.27	2.15	0.025
H2	**H2M3**	1.82	2.15	<0.001
H4	**H4M2**	2.28	1.55	<0.001
H5	**H5M3**	2.05	2.18	0.275
H6	**H6M2**	1.68	2.43	0.004
H6	**H6M4**	2.28	2.43	0.354
H6	**H6M5**	1.98	2.43	0.022
H7	**H7M1**	1.15	1.1	0.842
H7	**H7M2**	2.38	1.1	<0.001

[z] Diameter of colony was determined by culturing the 10th-generation mutants or parental isolates on potato dextrose agar 22ºC for one week. Each measurement was triplicated.

Table 2-3. Conidial production (10,000 unit/ml) of *Verticillium dahliae* mutants resistant to benzovindiflupyr compared to their parental isolates

Parental isolate	Mutant	Mutant conidial production [z]	Parental isolate (wild-type) conidial production	*P*
H1	**H1M2**	190	325	0.001
H2	**H2M1**	237	480	0.008
H2	**H2M3**	318	480	0.108
H4	**H4M2**	176	85	0.041
H5	**H5M3**	40	210	<0.001
H6	**H6M2**	165	183	0.702
H6	**H6M4**	718	183	0.041
H6	**H6M5**	170	183	0.743
H7	**H7M1**	605	475	0.321
H7	**H7M2**	245	475	0.004

[z] Conidia were counted using a 25x16 hemocytometer.

2.3.3.2. Competitiveness assay

Chi-square goodness-of-fit test showed that five out of six mutants had the same competitiveness as their parental isolates, while one mutant was different from its parent in competitiveness ($P < 0.01$). When the ratio of mutant and wild-type isolate was same (M:W = 50%:50%), the mutant H2M3 showed a higher competitiveness than its parent H2 (Table 2-4). When the mutant was at different population, H2M3 showed a lower competitiveness than H2 (Table 2-4).

Table 2-4. Germination ratios of mutants to wild-type isolates of *Verticillium dahliae* mutants under 5 µg/ml benzovindiflupyr

Mutant	Parental isolate	Expected			Observed			*P*
		25:75 [z]	50:50	75:25	25:75	50:50	75:25	
H2M1	**H2**	47.6	57.1	66.6	44.0	68.0	59.3	0.864
H2M3	**H2**	27.5	47.1	66.8	10.3	68.0	59.7	<0.01
H7M2	**H7**	40.5	58.4	76.4	39.3	70.3	65.7	0.701
H7M3	**H7**	2.9	5.9	8.8	7.3	4.0	6.30	0.993
H7M4	**H7**	45.0	61.4	77.9	57.0	55.3	72.0	0.561
H19M2	**H19**	18.1	36.1	54.2	26.3	59.7	22.3	0.096

[z] The ratio of mutants to wild type isolates (M:W).

2.3.3.3. Pathogenicity test

Through disease evaluation of inoculated potato plants, benzovindiflupyr-resistant *V. dahliae* mutants caused an average disease severity that ranged from 1.25 to 2.75 rating units compared to 1.5 to 2.75 for their parent isolates. Statistical analysis indicated mutants had the same virulence as their parental isolates (*P* ranged from 0.11 to 0.79) (Table 2-5).

Table 2-5. Pathogenicity of benzovindiflupyr mutants and their parental isolates on potato plants in greenhouse conditions

Parent isolates	Mutants	Disease severity caused by mutants [z]	Disease severity caused by parental isolates	*P*
H2	**H2M3**	2.50	2.25	0.79
H5	**H5M4**	2.75	1.75	0.11
H7	**H7M4**	1.25	1.5	0.54
H19M2	**H19M2**	2.25	2.75	0.55

[z] Disease severity was determined by categorizing disease symptoms on potato plant into five levels. 0: healthy, 1: discoloration in vascular system but no symptom on plant, 2: chlorosis of potato plant or slightly discoloration on tuber, 3: wilting of potato plant or discoloration on tuber, 4: dead potato plant.

2.3.3.4. Cross-resistance

No cross-resistance was shown between benzovindiflupyr and either boscalid, fluopyram, azoxystrobin, or pyrimethanil. The average EC_{50} of *V. dahliae* isolates and their benzovindiflupyr-resistant mutant to boscalid, azoxystrobin, mix of fluopyram and pyrimethanil, and fluopyram were 69.98, 0.76, 39.59, and 89.15, respectively. Most of the tested strains were resistant to boscalid/fluopyram, and the resistance did not change when sensitivity to benzovindiflupyr changed (ρ = -0.054, P = 0.876 and ρ = 0.218, P = 0.519 respectively) (Figure 2-6 A, D). For azoxystrobin, all strains tested were sensitive and did not increase resistance to azoxystrobin in the strains with a high resistance to benzovindiflupyr (ρ = -0.487, P = 0.128) (Figure 2-6 B). According to the statistical analysis, there also was no cross-resistance between benzovindiflupyr and pyrimethanil (ρ = -0.159, P = 0.640) (Figure 2-6 C).

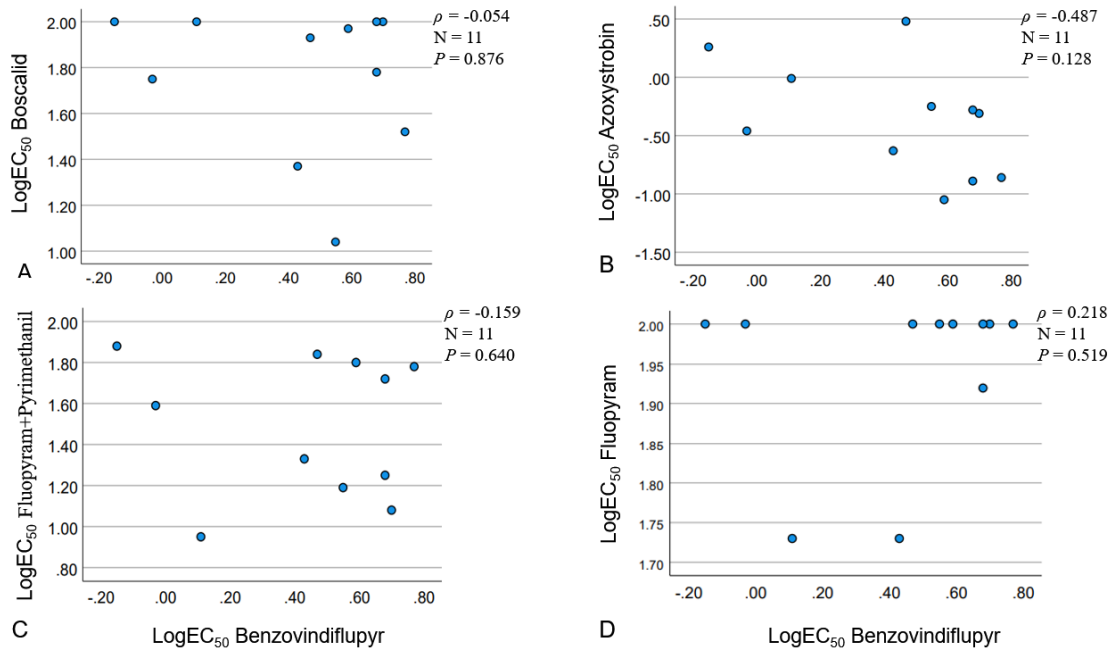

Figure 2-6. Cross-resistance of benzovindiflupyr-resistant *Verticillium dahliae* mutants, evaluated on their sensitivities to between benzovindiflupyr and A: boscalid, B: azoxystrobin, C: 25% fluopyram and 75% pyrimethanil, and D: fluopyram.

2.4. Discussion

For the 38 isolates of *V. dahliae*, a baseline sensitivity was generated, with EC_{50} ranging from 0.07 to 11.28 µg/ml, where 37% of them were distributed between EC_{50} of 0.51 and 1.00 µg/ml, and 89% showed EC_{50} <3.0. Three isolates with EC_{50} greater than 5 µg/ml were found. It indicated that natural resistance might have already occurred. Results of fitness test showed that most benzovindiflupyr-resistant mutants had a high level of adaptation. Therefore, frequent applications of benzovindiflupyr may result in a quick development of resistance and loss of fungicide efficacy. As such, the resistant population would become predominant.

There 18 benzovindiflupyr-resistant mutants obtained from eight *V. dahliae* isolates. Most of the mutants had high RF values and they were stable through generations. More importantly, they did not have significant trade off fitness and adaptation with resistance development, and they all maintained aggressive pathogenicity. Apparently, it can be a risk in the production. In fact, at least three isolates were found naturally resistant to benzovindiflupyr.

To reduce the risk, a practical way is to employ another fungicide that does not have cross resistance or have the same mode of action with benzovindiflupyr (Brent & Hollomon, 1998). By mixing or rotating applications, the probability that resistance occurs and becomes dominant is reduced (Brent & Hollomon, 1998). Azoxystrobin, a quinone outside inhibitor (QoI), had no cross resistance with benzovindiflupyr and was highly effective in inhibiting *V. dahliae*. Rotating application of azoxystrobin- and benzovindiflupyr-based fungicide or simply applying azoxystrobin and benzovindiflupyr combined fungicide such as Elatus can help to sustainably control PED in the field.

The mechanisms by which pathogens develop resistance may vary depending on the mode of actions of fungicides. Resistance controlled by fewer genes would result in a more consistent resistance with no significant change in RF values (Chen et al., 2019; Mao et al., 2018; Markoglou et al., 2006; Pang et al., 2013). The resistance against SDHIs is derived from mutations on different subunits, including SDHA, SDHB, SDHC, and SDHD, with mutations on the SDHB gene being the most common (Avenot et al., 2008, 2009; Sierotzki & Scalliet, 2013). The mutants to multi-site-target fungicides such as SDHI might have a varied resistance. For example, a study on the evaluation of resistance of *Didymella tanaceti* to SDHI fungicides reported that mutations at different sites led to different levels of resistance and can be classified by the RF values as low resistance (10 to 100), medium resistance (100 to 1000), and high resistance (>1000) (Pearce et al., 2019). Similar results were also observed in this study, where the RF values could be grouped into three clusters, with one being below 49, one between 50 and 249, and one above 1000. Therefore, molecular detection of mutation type may be worthwhile being carried out as the next step. As the mutation sites which lead to high resistance are addressed, it will be helpful for an integrated management.

Moreover, the multi-site-target feature of SDHI fungicides can also explain the great variation in the inhibitory effect of different SDHI fungicides on the same pathogen. Although

the mode of action is the same, different fungicides target on different sites and different pathogens have different sensitivities on different sites. For example, a study carried by Ishii et al. (2016) showed that tested *Colletotrichum* spp. isolates were sensitive to benzovindiflupyr while being completely insensitive to several other SDHI fungicides. This is similar to the data in the present study where boscalid and fluopyram, both SDHIs, showed very poor inhibition of *V. dahliae*. Therefore, although there is no cross resistance between different tested SDHI fungicides, it is still difficult to control PED by using another SDHI fungicide.

2.5. Conclusions

Verticillium dahliae had potential risk of resistance to benzovindiflupyr. This was based that benzovindiflupyr-resistant mutants have stable resistance and mostly possess equal or greater environmental fitness compared to their parents, suggesting a population of resistant isolates could overcome the effectiveness of benzovindiflupyr and be predominant. Therefore, strategic plans should be considered in fungicide applications.

CHAPTER 3

EFFECTS OF CHEMICAL AND BIOLOGICAL TREATMENTS ON POTATO EARLY DYING AND SOIL MICROBIOME

Chapter Abstract

Potato early dying (PED) is a soilborne disease caused by *Verticillium* spp., with *V. dahliae* being a predominant species. To control PED, chemical and biological products were studied, including Elatus (azoxystrobin and benzovindiflupyr), Aprovia (benzovindiflupyr), Stargus (*Bacillus amyloliquefaciens*), and Regalia (extract of *Reynoutria sachalinensis*) at Aroostook Farm, Presque Isle, ME in 2019 and 2020. Potato 'Superior' was grown, immediately followed by applying *V. dahliae* inoculum in the furrow. Test products were applied as a soil treatment after inoculum application, and Stargus and Regalia were also applied after row closure. Soil and plant roots were sampled, and DAN was extracted from the samples. Field plant diseases were evaluated before vine killing. Tuber disease and yield were measured after harvest. Quantitative polymerase chain reaction (qPCR) was performed using the extracted DNA as a template to quantify *V. dahliae*. Results showed that all products reduced plant disease incidences by 28% to 34% in 2019 and 20% to 43% in 2020, and reduced tuber disease incidences by 25% to 26% in 2019 and 21% to 41% in 2020 compared to non-treated plots. The population of *V. dahliae* was reduced in plant roots and soil by all these products. Soil microbial communities were analyzed by Illumina sequencing on V4 region of 16S rRNA gene for bacteria and ITS1 region for fungi. Fungal abundance at the family level varied, but less associated with beneficial or neutral plant interactions. Elatus treatment resulted in an increase in richness and alpha diversity of fungi one month after the application. Indigenous fungi were more likely to rebound quickly after soil treatment, while some pathogen populations remained low. Bacterial

communities were less impacted by fungicides. Thus, all the treatments were effective in PED control.

3.1. Introduction

The potato is the number one vegetable crop, and Maine is the ninth largest potato producing state in the U.S. (NASS, 2018). The crop is constrained by many biological stresses including potato early dying (PED). PED is caused by *Verticillium dahliae* and several other *Verticillium* spp. (Martin et al., 1982; Pegg & Brady, 2002). It can decrease potato yield by 10% to 15%, and up to 50% in severe situations (K Johnson et al., 1986; Powelson & Rowe, 1993).

Verticillium dahliae has a long life-span and wide range of hosts. The survival structure, microsclerotia, enables *V. dahliae* to stay viable in soil for over 14 years (Wilhelm, 1955), and more importantly, it can infect over 200 species of hosts (Berlanger & Powelson, 2005; Klosterman et al., 2009), which makes crop rotation less effective as a control strategy. Because the severity of PED is correlated with the amount of pathogen in soil (Li et al., 2017), PED control mainly focuses on reducing pathogen populations in soil and utilizing plant resistance. Some fungicides, such as azoxystrobin and benzovindiflupyr are effective and frequently used in potato production (G Bubici et al., 2006; Mihajlović et al., 2021; Syngenta, 2020; Wiglesworth & Tally, 2015). The mode of action of azoxystrobin is known as a quinone outside inhibitor (QoI) (Fernández-Ortuño et al., 2010), and that of benzovindiflupyr is a succinate dehydrogenase inhibitor (SDHI) (Avenot & Michailides, 2010).

Biologically based fungicides can also be candidates for PED control. *Bacillus* spp., mostly *B. subtilis*, *B. licheniformis*, and *B. velezensis* (formerly *B. amyloliquefaciens*) (Khan et al., 2018), are effective in the suppression of *Verticillium* spp. (Gomaa, 2012; B. Li et al., 2013; S. Li et al., 2013). The mode of actions of these products include direct inhibition of plant pathogens, nutrient and space competition, and induced localized or systemic resistance, and growth promotion of the plant (Dicklow & Madeiras, 2018). Plant extracts have been shown to effectively control *V. dahliae* by inducing plant defense (Uppal et al., 2008; Romera et al., 2019). Extract of *Reynoutria sachalinensis* is an example of this type of product (Osborne et al., 2009).

Fungicides that have a mode of action as respiration inhibitors have a broad-spectrum target. They not only suffocate pathogens, but also can poison natural or beneficial organisms. Therefore, the entire soil microbiome, consisting of bacteria, fungi, and archaea, can be impacted (J. Li et al., 2017; Sederholm et al., 2018). It is not clear how applied fungicides affect soil microbiome.

The objectives of this study were to investigate the effect of chemical and biological products on *V. dahliae* populations, PED, and related yield of potato through field trials. Additionally, the changes in soil microbial communities under chemical fungicide treatment will be examined in order to understand the impact of soil treatments.

3.2. Materials and Methods

3.2.1. *Verticillium dahliae* isolates

Nineteen *V. dahliae* isolates were obtained from diseased potato stems collected in Maine, previously by Lambert lab, University of Maine. Isolation was performed by cutting the wilted stems with microsclerotia as well as discolored tubers into 0.5-cm-long pieces, disinfesting with 75% ethanol for 30 seconds followed by 10% bleach for 5 min, and immediately rinsing with sterile tap water. The disinfested stem pieces were placed onto the semi-selective medium NP-10 in a Petri plate (Kabir et al., 2004). Pure culture of each isolate was derived using a single spore method on PDA plates. Genomic DNA of *V. dahliae* isolates was extracted, which was used as a template for performing polymerase chain reaction (PCR) targeting the ITS region. The PCR products were sequenced, which were analyzed using basic local alignment search tool (BLAST) algorithm against the National Center for Biotechnology Information (NCBI) database for positive identification.

3.2.2. Soil treatment materials

Fungicides Aprovia (active ingredient or a.i. 10.3% benzovindiflupyr) and Elatus (a.i. 30.0% azoxystrobin and 15.0% benzovindiflupyr) (Syngenta, Greenboro, NC, United States)

were selected for both 2019 and 2020 field trials. Additionally, Stargus (a.i. 96.4% $>10^9$ cfu/ml *Bacillus amyloliquefaciens*) and Regalia (a.i. 20% extract of *Reynoutria sachalinensis*) were used as biological fungicides in the 2020 field trial.

3.2.3. Inoculum preparation

Inoculum of *V. dahliae* was prepared using oat seed as a medium. Six liters of oat seeds were placed in a 12-liter filtered mushroom growing bag and autoclaved at 121°C for 60 min twice in a 24-h interval. Ten plugs of fresh cultured *V. dahliae* isolates were cut by a 6-mm cork borer and transferred into the bag, which was then sealed immediately. The culture was incubated at room temperature for at least four weeks. During incubation, the bags were shaken every other day for better inoculum distribution and aeration. After the incubation, the inoculated oat seed was air dried and stored at 4 °C until use.

3.2.4. Field trials

Field trials were conducted at Aroostook Research Farm, located in Presque Isle, ME in 2019 and 2020. In the 2019 trial, each plot consisted of three 25-ft-long and three-ft-wide rows with 1-ft plant spacing. *Verticillium dahliae* inoculum was evenly spread in the furrows at the rate of 12.5 ml/foot and potato variety 'Superior' seed pieces were hand planted on May 24th. Fertilizer (N:P:K = 14:14:14) was applied at planting at 1,100 lb/A. Test products included Elatus (a.i. azoxystrobin and benzovindiflupyr; Syngenta, Greensboro, NC, United States), Aprovia (a.i. benzovindiflupyr; Syngenta), Stargus (*Bacillus amyloliquefaciens*; Marrone Bio-Innovations, Davis, CA, United States) and Regalia (extract of *Reynoutria sachalinensis*; Marrone). Elatus at 280.9 ml/A and Aprovia at the rate of and 303.5 ml/A were applied in the furrow at planting. Non-chemical applied plots (NT) were set as a positive control group, while non-chemical non-inoculation (NTNI) plots were set as negative control. The trial was arranged in a randomized complete block design with four replications.

On June 24th emergence was determined by counting those emerged out of 50 planted potatoes in 2 rows. Emergence rate was calculated as (the number of emerged plants / number of total number of seed) x 100%. Evaluation of plant symptoms was performed on August 20th by counting the number of symptomatic plants in the middle two rows and calculated as (number of symptomatic plants / number of total plants) x 100% (Figure 3-1B). Potato vines were killed using Reglone (a.i. diquat dibromide) at 1.5 lb/A on 4th and 11th of September. Potato tubers in the middle two rows of each plot were harvested by a one-row harvester on September 14th. The harvested tubers were washed and weighed, and the severity of tuber disease was rated on October 7th. A total of 50 tubers were randomly selected to evaluate severity of diseases by measuring the percentage of tuber lesions by cutting the tuber into two parts and observing the symptoms (Figure 3-1A).

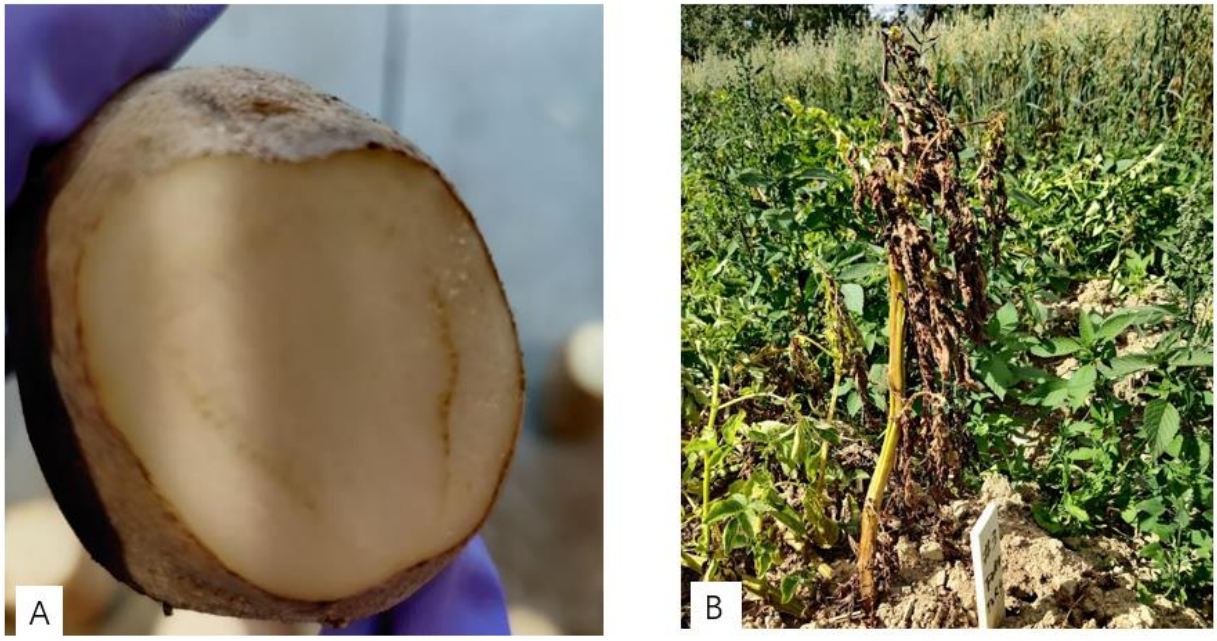

Figure 3-1. Symptoms of potato early dying showing vascular discolored ring on potato tuber (A) and wilt or flagging dying symptoms on potato plant (B).

In 2020, a field trial was conducted and operated as described above with additional treatments added. Treatments included applying 1) Elatus at 280.9 ml/A in the furrow at planting; 2) Aprovia at 303.5 ml/A in the furrow at planting; 3) Stargus at 0.5 gal/A in furrow at planting for the first time and 1 gal/A by soil drench at emergence for the second time; and 4) Regalia at 2.7 gal/A in furrow at planting and 0.25% v/v in water on foliage one month after planting and again 10 days after that. Non-chemical (NT) and non-chemical non-inoculation (NTNI) plots were set as controls. Potato variety 'Superior' seed pieces were hand placed in furrow on May 27th. Emergence was counted and calculated on July 2nd. Evaluation of plant symptom was performed on September 5th. Potato vines were killed on 15th and 21st of September. Potato tubers in the middle two rows of each plot were harvested by a one-row harvester on September 25th. The harvested tubers were washed and weighed, and the severity of tuber disease was rated on October 9th.

Soil was sampled by collecting a minimum of 15 core samples from two rows in each plot using a 6-inch hand trowel. Soil sampling was done at planting (May 27th in 2019 and May 25th in 2020), one month after planting (July 3rd in 2019 and June 29th in 2020), and at harvest (20th in 2019 September 5th in 2020). In addition, on June 29th, 2020, potato plants were dug out and rhizosphere soil and roots were collected by shaking off the excess soil. The collected soil was immediately put in an iced cooler and transported to the lab. The soil was sieved through a 10-mesh (2 mm) sieve to remove the rocks and weeds, then stored in 15 ml centrifuge tubes in a -80°C freezer.

3.2.5. Soil DNA extraction

Genomic DNA was extracted from 0.25 g sampled soil from each plot using the DNeasy PowerSoil Pro Kit (QIAGEN Inc., Germantown, MD, United States) following the manufacturer's instruction. DNA concentration was quantified with a NanoDrop 2000 (Thermo Fisher Scientific, Wilminton, DE, United States). Each DNA sample was measured three times

and the mean value was used. PCR was performed using ITS1 and ITS4 primer pairs to confirm the DNA quality.

3.2.6. Quantification of *Verticillium dahliae* via qPCR

The quantification of *V. dahliae* was performed by qPCR. A pair of *V. dahliae*-specific primers was used targeting on the β-tubulin gene (VertBT F/VertBT R) (Atallah et al., 2007). Reaction mix was prepared by adding 1 µl DNA, 10 µl Luna® Universal qPCR Master Mix (New England Biolabs Inc., Ipswich, MA, United States), 0.5 µl forward primer (VertBT_F 5′-AACAACAGTCCGATGGATAATTC3′) and reverse primer (VertBT_R 5′-GTACCGGGCTCGAGATCG-3′), and adjusted to 20 µl with DNase-free. The PCR was performed on Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States) using the settings: starting at 95℃ for 3 min, followed by 40 cycles of 95℃ for 10 sec and 63℃ for 35 sec (Aljawasim & Vincelli, 2015). To calculate the quantity of *V. dahliae* in soil, a standard curve was established by amplifying pure *V. dahliae* DNA at 20 ng/ul, 2 ng/ul, 0.2 ng/ul 0.02 ng/ul and 0.002 ng/ul.

3.2.7. Soil microbiome analysis

3.2.7.1. Bacterial community

Soil DNA samples were amplified by PCR using primer pair 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACHVGGGTWTCTAAT) to the (Kozich, 2013). The reaction was in a 25 µl volume, which consisted of 5 µl of 5x Green GoTaq reaction buffer, 0.5 µl of dNTPs, 0.5 µl of each pair of primers, 0.13 µl of GoTaq DNA Polymerase, 1 µl of DNA, and 17.4 µl of DNase-free water. Thermal cycler setting consisted of an initial denaturation of 3 min at 95°C, followed by 30 cycles of 45 sec at 95°C, 60 sec at 50°C and 90 sec at 72°C, with a final elongation of 10 min at 72°C. PCR amplicons were examined by gel electrophoresis (2%) to ensure they were the expected size. DNA from Elatus treated and non-fungicide treated plots were diluted into 4 ng/ul and submitted to Michigan State University

Genomic Core Facility (East Lansing, MI, United States) for Illumina sequencing. Submitted DNA was amplified by same Illumina compatible amplicon libraries of the 16S rRNA V4 hypervariable region using barcoded primer pair 515f/806r, followed by sequencing via Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) using a 2 x 250 bp paired end format.

3.2.7.2. Fungal community

The two-step PCR method was employed. The first step was performed using ITS1/ITS2 primer pair with tags on the 5' ends (CS1-ITS1: ACACTGACGACATGGTTCTACA – TCCGTAGGTGAACCTGCGG; CS2-ITS2: TACGGTAGCAGAGACTTGGTCT - GCTGCGTTCTTCATCGATGC). The reaction was in a 25 µl volume using the same formulation as bacterial amplification. Thermal cycler setting consisted of an initial denaturation of 2 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 53°C and 60 sec at 72°C, with a final elongation of 10 min at 72°C. A gel electrophoresis (2%) was run to confirm the amplicons were at the expected size, and off-target products were not present. All PCR amplicons were diluted into 4 ng/ul and submitted to Michigan State University Genomic Core Facility (East Lansing, MI, United States) for Illumina sequencing. Submitted samples were then amplified by Illumina compatible amplicon tags using barcoded primer pair CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT), followed by sequencing via Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) using a 2 x 250 bp paired end format.

3.2.8. Data analysis

Data were analyzed using SAS studio (SAS Institute, Cary, NC, United States). General linear model (GLM) was used for examining treatment effects. The Student-Newman-Keul test ($\alpha = 0.05$) and Fisher's LSD test ($\alpha = 0.05$) were used for the analysis of emergence rate, disease incidences, and yield. The Illumina sequencing data were analyzed using the MOTHUR software

pack (version 1.39.5, https://mothur.org/). Operational taxonomic unit (OTU) was used to group microbes based on 97% similarity (Schloss et al., 2009). Sequences were processed according to the MiSeq SOP (https://mothur.org/wiki/miseq_sop/), including reducing sequences and PCR errors, finding unique sequences, aligning sequences to the Silva reference database for bacteria and to the UNITE reference database for fungi (UNITE, 2019; Yilmaz et al., 2014), and assessing error rates. The output was then processed in RStudio (http://www.rstudio.com/) for visualization of data. Relative abundance at 0.5% and 2% was used as a cut-off point for bacterial and fungal community analysis at the family level, respectively.

3.3. Results

3.3.1. Emergence evaluation

In the 2019 field trial, the highest emergence rates were found in both Elatus- and Aprovia-treated plots, both of which were significantly higher than the NT plots, with the Elatus-treated plots being the highest and the Aprovia treated plot being the second. Emergence rates in the NTNI plots were not significantly different from either the NT plots or the two fungicide plots (Table 3-1).

The emergence rate in 2020 was much lower than in 2019. The highest emergence rate was only 46%, which was one third less than the lowest emergence in 2019. There were no significant differences in emergence rate between treatments, all ranging from about 40% to 45%, except for the Aprovia treated plots, which were as low as 33% (Table 3-2). Due to the low emergence rate, an arial view photo was used to compare the differences between positions (Figure 3-2). The SRR (combination of Stargus and Regalia and applied Regalia again) plot emergence rate at the top row was significantly lower than the other SRR plots; the NTNI (non-treated non-inoculated) plot on the left of the top row had minimal emergence; similarly, on the right of the top row, Elatus (applied Elatus) and NT plots (non-treated) also showed lower emergence than the other plots of the same treatments (Figure 3-2).

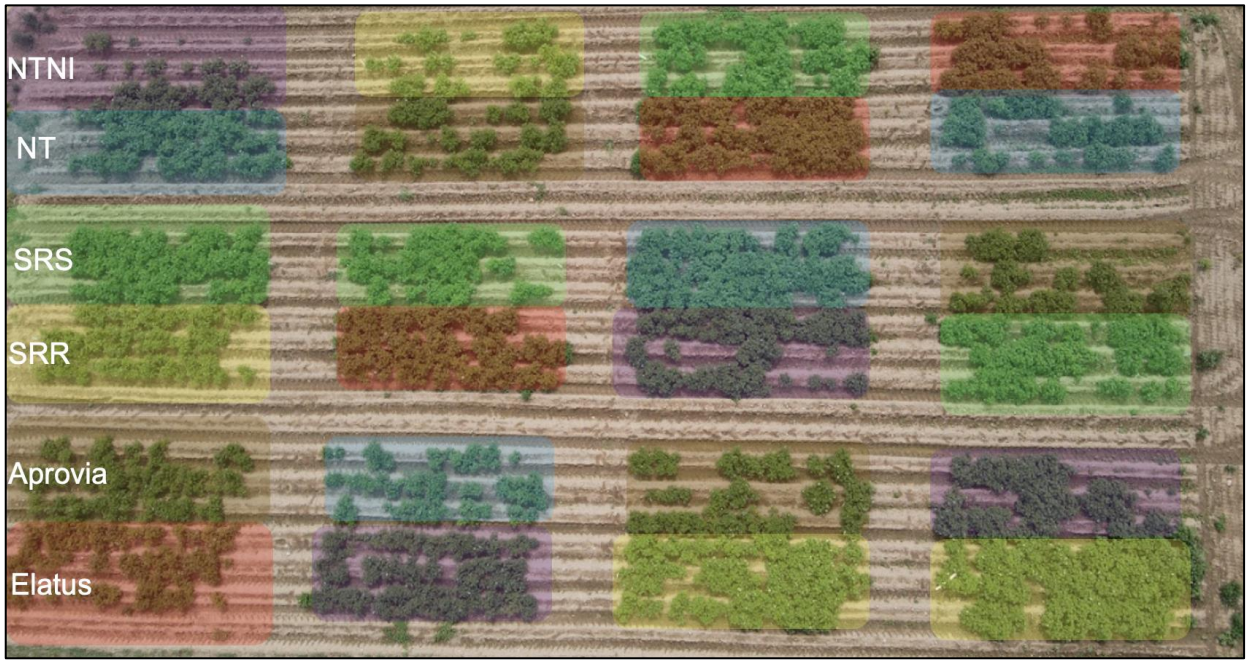

Figure 3-2. Aerial view of field trial plot arrangement in 2020. Photo was taken on August 4th, 2020. Different treatments were masked with different colors: Elatus at 280.9 ml/A, Aprovia at 303.5 ml/A, combination of 0.5 gal/A Stargus and 2.7gal/A Regalia at planting and Regalia at 0.25% v/v on foliar at emergence followed by same foliar application of Regalia 10 days later (SRR), combination of 0.5 gal/A Stargus and 2.7 gal/A Regalia at planting and 1 gal/A Stargus soil drench at emergence (SRS), non-treated (NT) for *Verticillium dahliae* infested soil, and non-treated and non-inoculated (NTNI).

3.3.2. Disease incidence and yield assessment

In 2019, field plant disease incidences in plots with treatment of either Elatus or Aprovia was 34% and 28% lower than the NT plots and 116% and 136% higher than the NTNI plots, respectively (Figure 3-3A). A similar pattern was observed in the tuber disease incidence evaluation. The lowest values were found in the NTNI plots, followed by Elatus, and Aprovia, with NT plots showing the highest disease levels. However, tuber disease incidences were not statistically different between groups. Elatus-treated plots showed the highest numerical yields,

with values 19% higher than NT plots, whereas Aprovia treated plots showed the lowest numerical yield, but yield differences were not statistically significant (Table 3-1).

In 2020, the highest and lowest field plant disease incidences belonged to NT and NTNI plots, respectively, and they were significantly different from each other. All of the fungicide-applied plots decreased the disease symptoms by 41%, 21%, 34%, 29% in Elatus-treated, Aprovia, the SRR, the SRS plots, respectively. The combination of Stargus and three-applications of Regalia (SRR) had the overall best performance on disease suppression (Figure 3-3B). Yield under the different treatments ranged from 118.79 to 161.76 cwt/A, where the highest yield was observed in SRR plot and the nominally lowest in low-emergence plot—Aprovia, although there were no significant differences in yield among treatments (Table 3-2).

Table 3-1. Emergence and yield of potato in 2019

Treatment	**Application time**	**Soil infestation** [z]	**Emergence (%)**	**Yield (cwt/A)**
Elatus (@ 280.9 ml/A)	At planting	Yes	96 a [y]	181.32 a
Aprovia (@ 303.5 ml/A)	At planting	Yes	94 a	146.38 a
Non-treated	-	Yes	79 b	152.34 a
Non-treated	-	No	82 ab	168.11 a

[z] Inoculum *Verticillium dahliae* grown on oat seed was applied at 20 g/foot plot. [y] Column numbers followed by the same letter are not significantly different at $\alpha = 0.05$ as determined by Student-Newman-Keuls test.

Table 3-2. Emergence and yield of potato in 2020

Treatment	Application time [z]	Soil infestation [y]	Emergence (%)	Yield (cwt/A)
Elatus (@ 280.9 ml/A)	A	Yes	42 a [x]	141.25 a
Aprovia (@ 303.5 ml/A)	A	Yes	33 a	118.79 a
Stargus (@0.5 gal/A) Regalia (@2.7 gal/A at A; 0.25% v/v at B and D)	A ABD	Yes	43 a	161.76 a
Stargus (@0.5 gal/A at A; 1gal/A soil drench at C) Regalia (@2.7 gal/A)	AC A	Yes	44 a	132.88 a
Non-treated	-	Yes	46 a	143.65 a
Non-treated	-	No	41 a	145.41 a

[z] Application time: A: in furrow at planting; B: on foliar at emergence; C: soil drench at emergence; D: 10 days after B. [y] Inoculum *Verticillium dahliae* grown on oat seed was applied at 20 g/foot plot. [x] Column numbers followed by the same letter are not significantly different at $\alpha = 0.05$ as determined by Student-Newman-Keuls test.

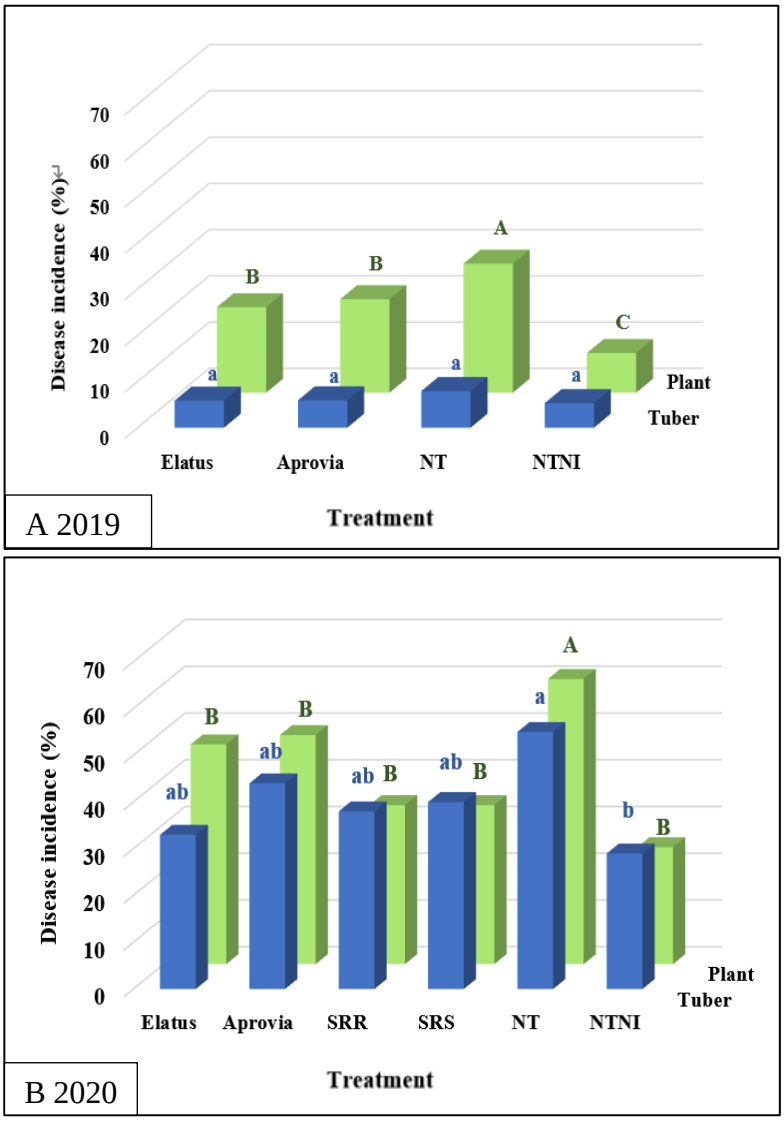

Figure 3-3. Disease incidence on plant and tuber potato in field trials in 2019 (A) and 2020 (B) Treatments included 1) Elatus at 280.9 ml/A, 2) Aprovia at 303.5 ml/A, 3) combination of 0.5 gal/A of Stargus and 2.7gal/A of Regalia at planting and Regalia at 0.25% v/v foliar application at emergence followed by same foliar application of Regalia 10 days thereafter (SRR), 4) combination of 0.5 gal/A Stargus and 2.7 gal/A Regalia at planting and 1gal/A Stargus applied as a soil drench at emergence (SRS), 5) non-treated (NT) for *Verticillium dahliae* infested soil, and 6) non-treated and non-inoculated (NTNI). Significance was determined by Student-Newman-Keuls test ($\alpha = 0.05$).

3.3.3. *Verticillium dahliae* quantification

In 2019, the NT plots started with a higher population of *V. dahliae* at planting but decreased throughout the season. Similarly, the NTNI plots had the same trend but with a lower overall *V. dahliae* population. At all three sampling times, both Elatus and Aprovia reduced *V. dahliae* in soil, with some exceptions (Figure 3-4A).

In 2020, a similar trend was observed. The numbers of *V. dahliae* in the SRS, NT, NTNI plots significantly increased after two weeks. However, NT plots had an unexpectedly low number of *V. dahliae* at planting. One month post planting, the lowest numbers were observed in the NTNI plots, while the highest numbers were observed in the SRS plots followed by Elatus-treated plots (Figure 3-4B). Compared to the inconsistency in soil samples, the data in root soil samples were more consistent with expectations and showed a lower standard error. The highest numbers of *V. dahliae* were observed in the NT plots while the lowest were in the NTNI plots. Among four fungicide-treated plots, Aprovia-treated plots and the SRR plots had lower *V. dahliae* numbers than Elatus-treated and the SRS plots (Figure 3-4B).

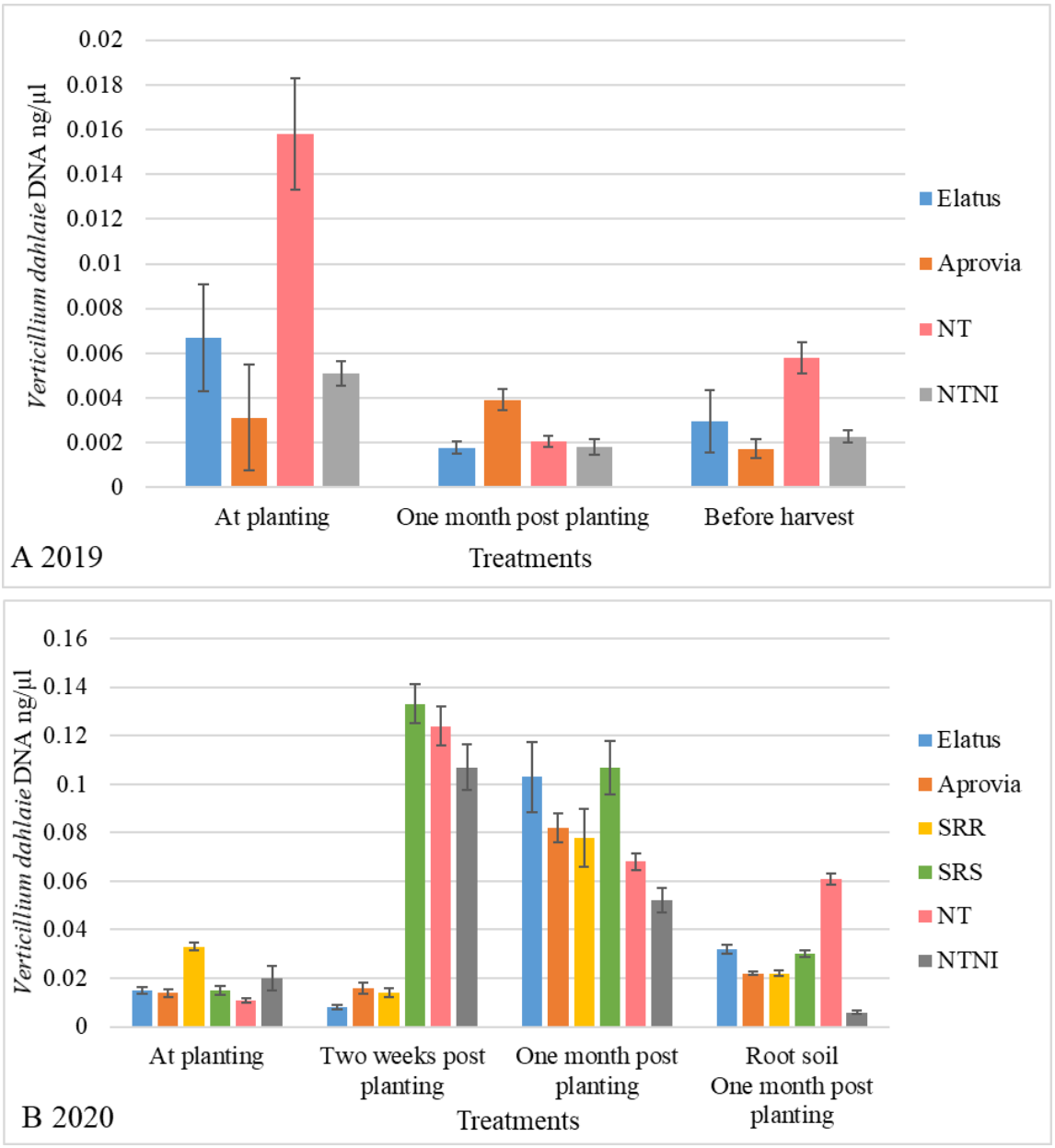

Figure 3-4. Quantitative estimation of *Verticillium dahliae* in soils of 2019 (A) and 2020 (B) treated with 1) Elatus at 280.9 ml/A, 2) Aprovia at 303.5 ml/A, 3) combination of 0.5 gal/A Stargus and 2.7gal/A Regalia at planting and Regalia at 0.25% v/v on foliar at emergence followed by same foliar application of Regalia 10 days later (SRR), 4) combination of 0.5 gal/A Stargus and 2.7 gal/A Regalia at planting and 1gal/A Stargus soil drench at emergence (SRS), 5) non-treated (NT) for *Verticillium dahliae* infested soil and 6) non-treated and non-inoculated (NTNI). Data was analyzed by quantitative polymerase chain reaction. Error bar was used for significance.

3.3.4. Bacterial community changes under Elatus application

A total number of 1,254,831 reads and 21,516 OTUs were obtained from 16S amplicon sequencing in 2019. According to observed OTUs, Chao1, and ACE estimators, Elatus-treated plots showed a relatively lower bacterial richness at planting. One month later, although richness decreased in both Elatus-treated plots and the NT plots, Elatus-treated plots had a higher richness than NT plots (Figure 3-5A). Shannon diversity showed that Elatus-treated plots and NT plots had a similar diversity at both time points, with a decrease in diversity being observed after one month of potato growth (Figure 3-5A).

A total of 33 families were classified in 2019. All families are listed in the Figure 3-6, and all microorganisms that could not be classified were grouped as "Other". One month after the application of Elatus, families Chitinophagaceae, Geobacteraceae, Nocardiaceae, Nocardioidaceae, Phyllobacteriaceae, Pseudomonadaceae, Sphingobacteriaceae, Sterptomycetaceae, Xanthomonadaceae slightly increased in their abundance, while Polyangiaceae slightly decreased. Families that decreased in abundance one month after planting compared to at planting were Gemmatimonadaceae, Sphingomonadaceae, Planctomycetaceae, while families Caulobacteraceae, Rhizobiaceae, Paenibacillaceae, Oxalobacteraceae increased (Figure 3-6).

In 2020, a total number of 983,874 reads and 20,182 OTUs were obtained. Observed OTUs, Chao1, and ACE estimators showed a similar richness for both Elatus-treated plots and the NT plots at planting, and they both dropped after one month (Figure 3-5B). Shannon diversity estimator indicated that Elatus-treated plots and the NT plots had a similar diversity at planting, but the diversity in Elatus-treated plots dropped after one month (Figure 3-5B).

In 2020, there were 36 families that were classified, excluding the non-classified "Others". The families with increased abundance in both Elatus-treated and NT plots one month

post planting were Alicyclobacillaceae, Geodermatophiaceae, Mycobacteriaceae, Nocardioidaceae, Oxalobacteraceae, Rhizobiaceae, Sphingomonadaceae, Sterptomycetaceae, Xanthomonadaceae, while the abundance of Bradyrhizobiaceae, Hyphomicrobiaceae, Micrococcaceae, Phyllobacteriaceae, Planctomycetaceae decreased (Figure 3-7). One month after the application of Elatus, families with a greater abundance increase than that of NT plots were Enterobacteriaceae, Nakamurellaceae, Sphingobacteriaceae, while Methylobacteriaceae decreased (Figure 3-8).

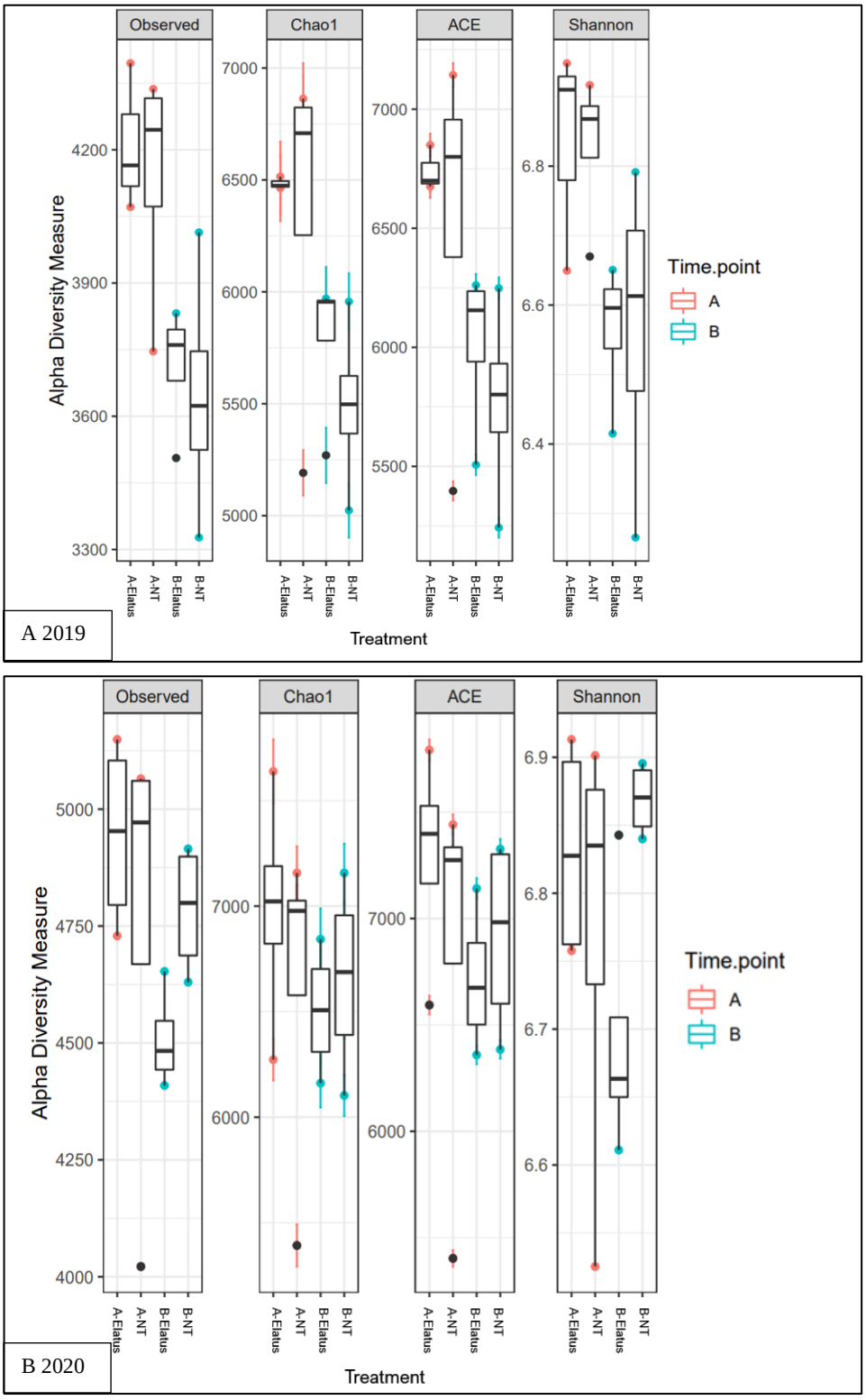

Figure 3-5. Bacterial alpha diversity in soils in 2019 (A) and 2020 (B). Time points included A: at planting; B: one month after planting. Treatments included Elatus applied at 280.9 ml/A and Non-treated (NT). Analyses were performed using observed OTUs, Chao1 index, ACE index, and Shannon diversity index.

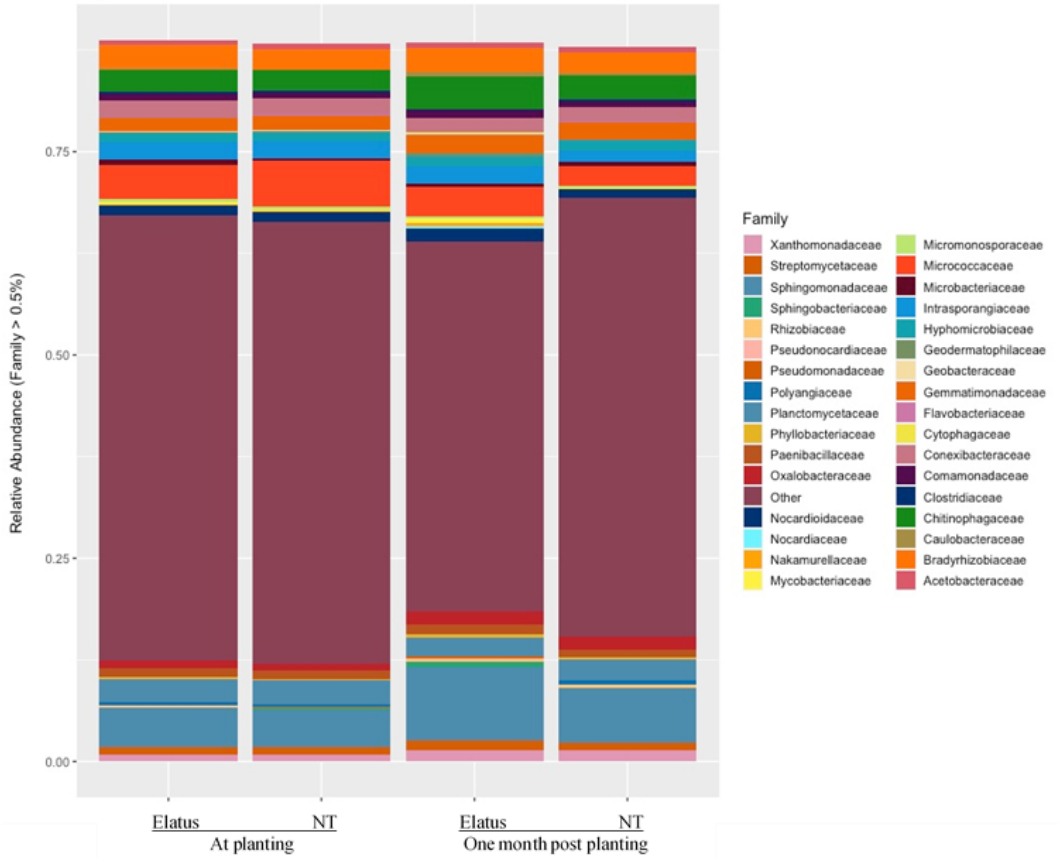

Figure 3-6. Relative abundances of bacteria at the family level in soils at different time points under fungicide applications in 2019. Time point A: At planting; B: One month post planting. Treatments included Elatus applied at 280.9 ml/A and Non-treated (NT). Taxa with relative abundance < 0.5% were excluded. Each color represents a different bacterial family.

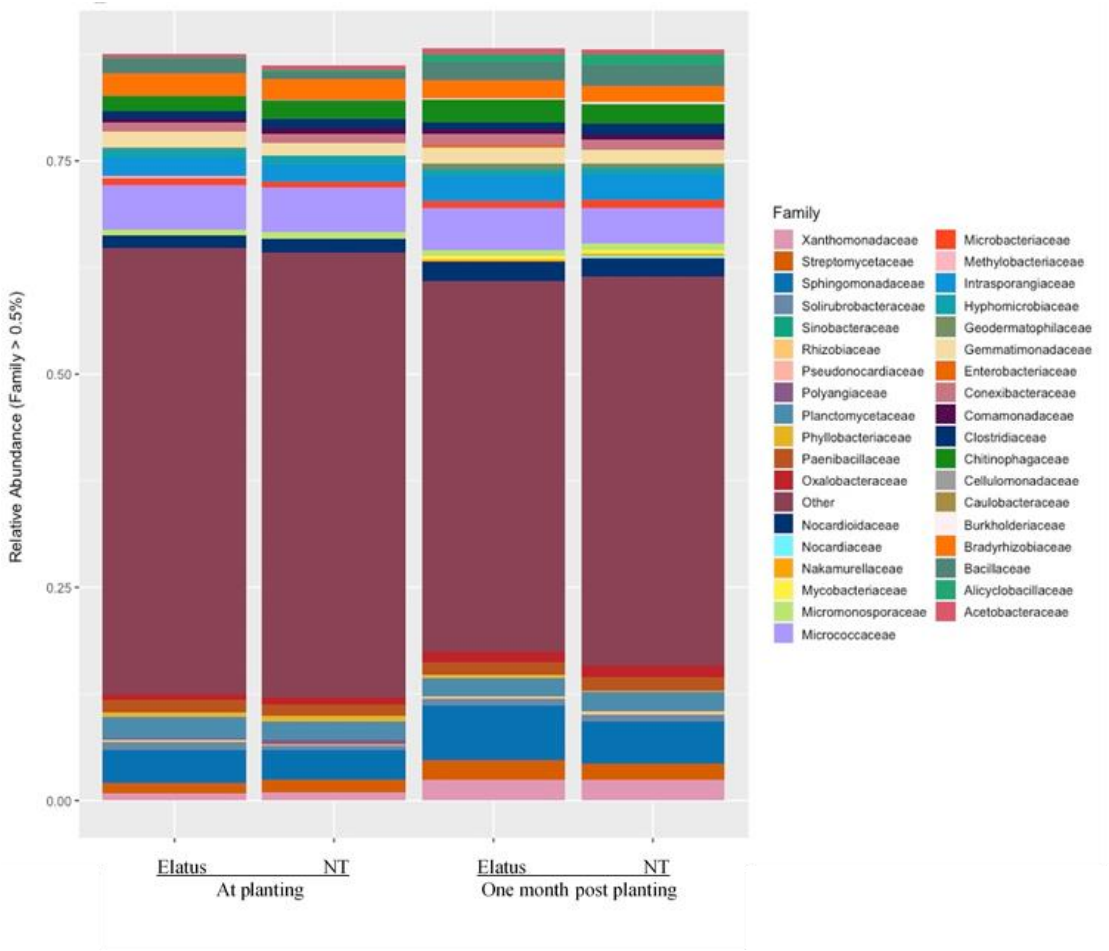

Figure 3-7. Relative abundances of bacteria at the family level in soils at different time points under fungicide applications in 2020. Time point A: At planting; B: One month post planting. Treatments included Elatus applied at 280.9 ml/A and Non-treated (NT). Taxa with relative abundance < 0.5% were excluded. Each color represents a different bacterial family.

3.3.5. Fungal community changes under Elatus application

In 2019, a total number of 1,330,476 reads and 7705 OTUs were obtained from ITS amplicon sequencing. Observed OTUs and two richness estimators Chao1 and ACE indicated NT plots had a higher richness at planting, but richness dropped one month after planting. The richness in Elatus-treated plots increased one month after planting (Figure 3-8A). Shannon diversity index showed a similar trend, as the highest diversity was shown in NT plots at planting, and the Eltaus-treated plots showed a higher diversity one month post planting (Figure

3-8A). Overall, the NT plots decreased in their richness and diversity as potatoes grew, while the Elatus-treated plots increased.

A total of 23 families were classified in 2019 as shown in the Figure 3-9. All microorganisms that could not be classified were grouped as "Other". Abundance increased in both Elatus-treated and NT plots one month post planting for families Hydnodontaceae, Trichomeriaceae, Tubeufiaceae, while decreased for Aspergillaceae, Periconiaceae (Figure 3-9). Compared to NT plots, abundance increased after the application of Elatus for families Hydnodontaceae, Mortierellaceae, Mrakiaceae, Piskurozymaceae, Phaeospaeriaceae, Pseudeurotiaceae, Rhizopodaceae, while decreased for Amniculicolaceae, Chaetomiaceae, Cucurbitariaceae, Didymellaceae, Didymosphaeriaceae, Ganodermataceae, Helotiaceae, Melanommataceae, Periconiaceae, Trichomeriaceae (Figure 3-9).

In 2020, a total number of 1,195,107 reads and 5443 OTUs were obtained. Observed OTUs, Chao1, and ACE estimators showed a higher richness in Elatus-treated plots in both the time points at planting and one month post planting. Fungal richness declined in the NT plots as potatoes grew (Figure 3-8B). Shannon diversity index indicated that Elatus-treated plots had a relatively higher diversity at planting and a relatively lower diversity one month post planting (Figure 3-8B).

A total of 28 families were identified in 2020, excluding the non-classified "Others". The families with increased abundance in both Elatus-treated and NT plots one month post planting were Piskurozymaceae, Pleosporaceae, Rhizopodaceae, Sonoraphlyctiadaceae, while abundance of Aspergillaceae, Bulleribasidiaceae, Helotiaceae, Mortierellaceae, Mrakiaceae, Mycosphaerellaceae, Myxotrichaceae, and Pseudeurotiaceae decreased. One month post planting, the Elatus-treated plots showed families with a greater increased abundance than that of NT plots were Herpotrichiellaceae, Microdochiaceae, Trichocomaceae, while Corticiaceae, Phaeosphaeriaceae decreased in their abundance (Figure 3-10).

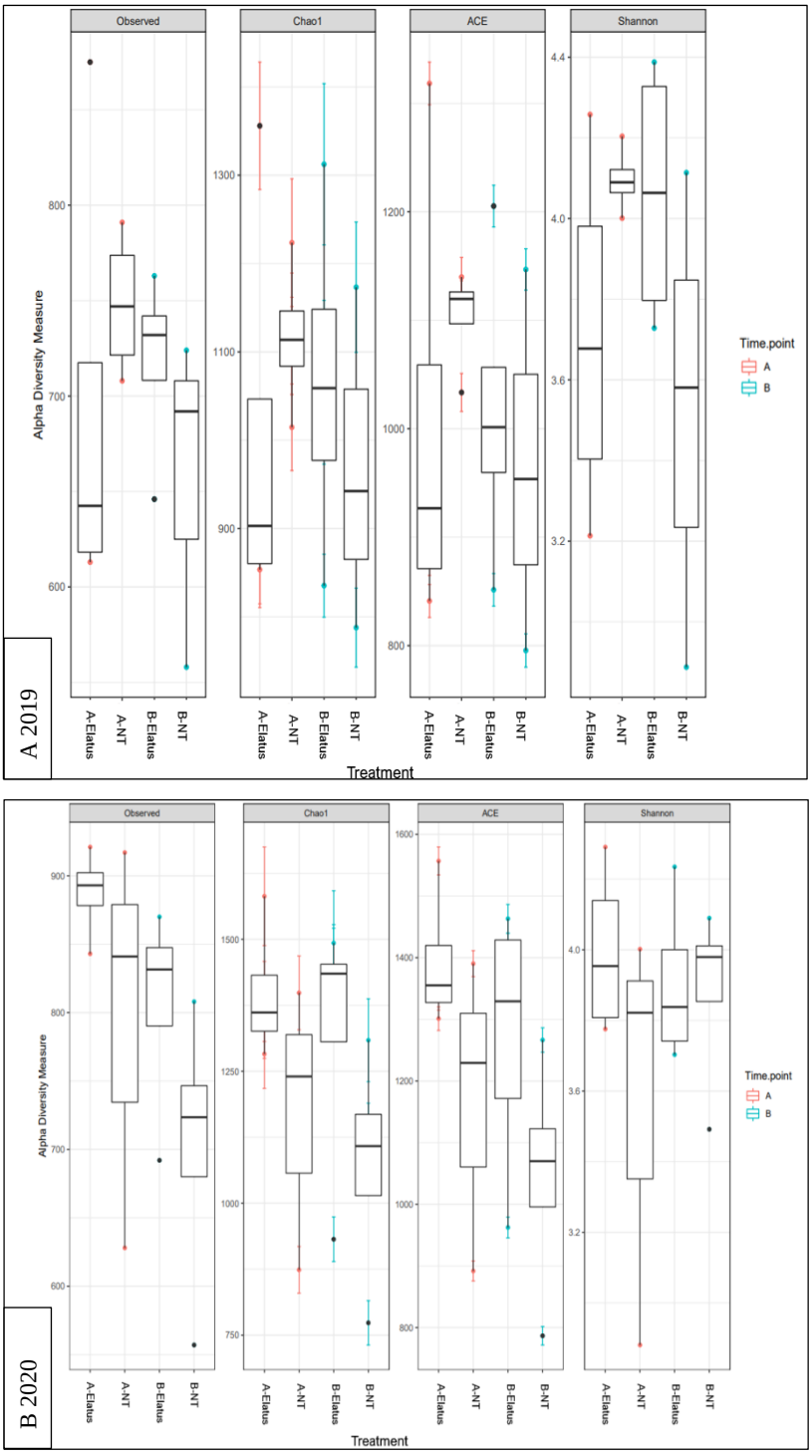

Figure 3-8. Fungal alpha diversity in soils in 2019 (A) and 2020 (B). Time points included A: at planting; B: one month after planting. Treatments included Elatus applied at 280.9 ml/A and Non-treated (NT). Analyses were performed using observed OTUs, Chao1 index, ACE index, and Shannon diversity index.

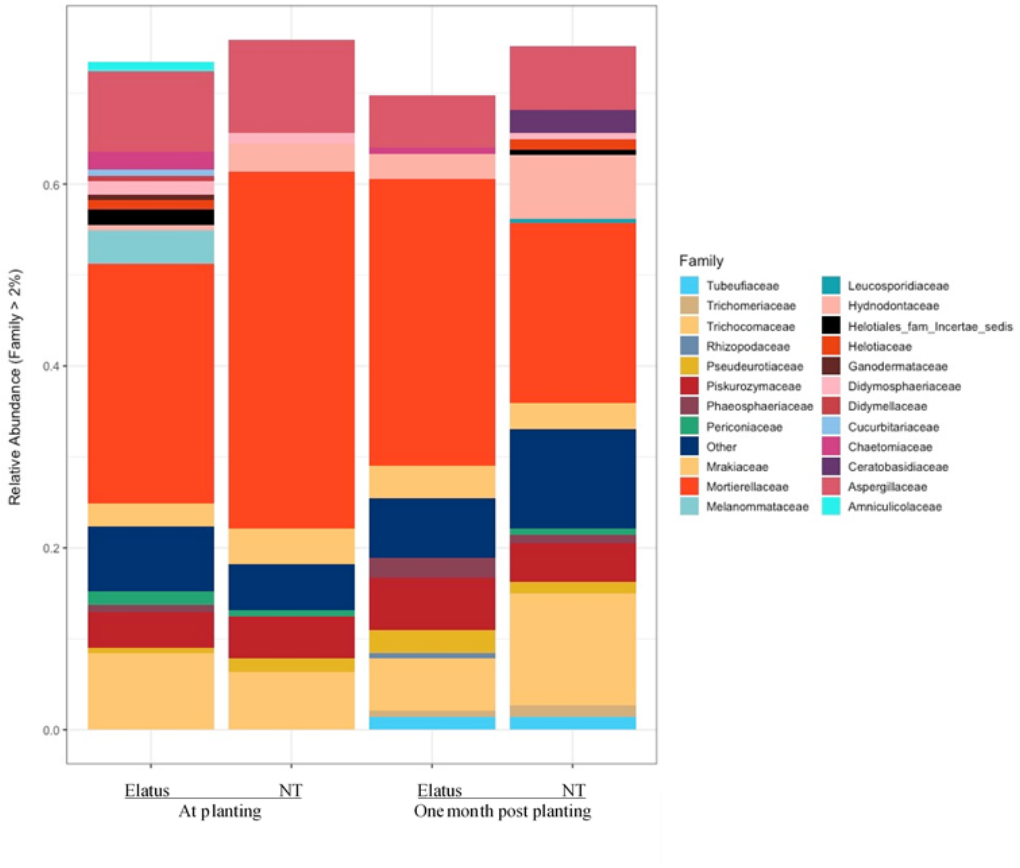

Figure 3-9. Relative abundances of fungi at the family level in soils at different time points under fungicide applications in 2019. Time point A: At planting; B: One month post planting. Treatments included Elatus applied at 280.9 ml/A and Non-treated (NT). Taxa with relative abundance < 2% were excluded. Each color represents a different fungal family.

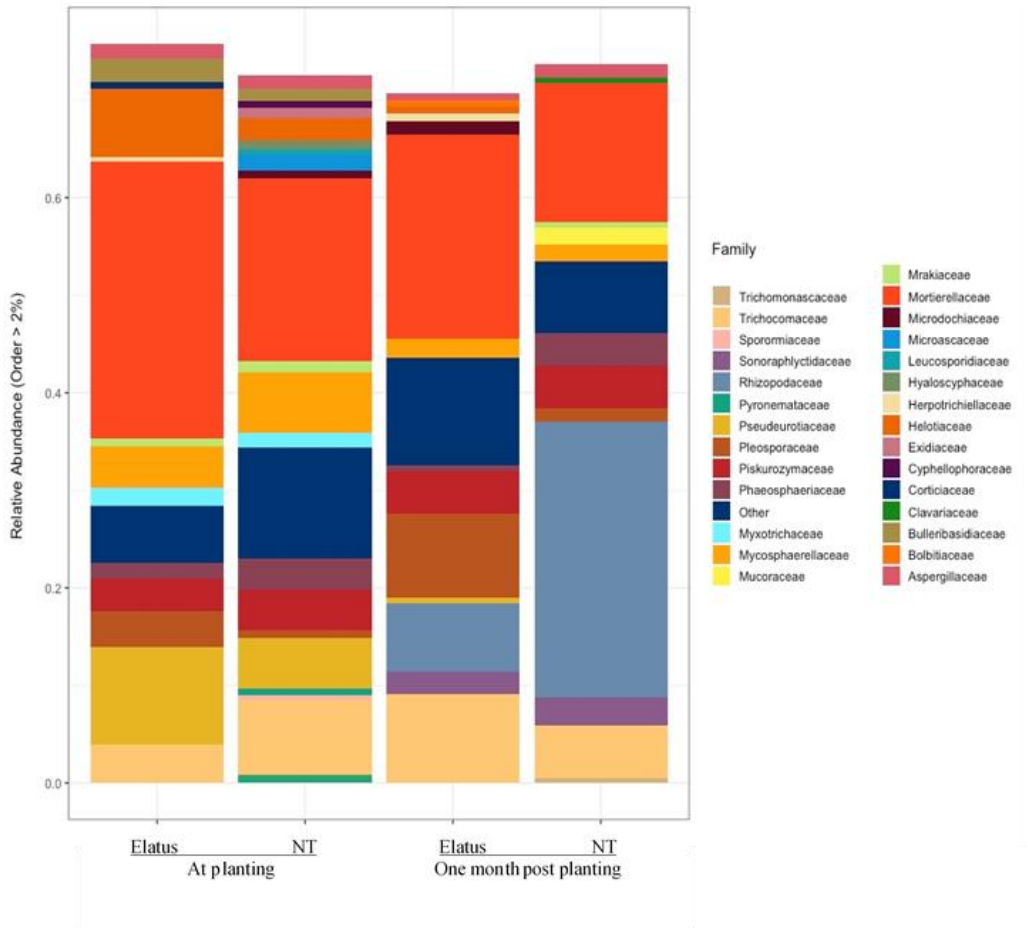

Figure 3-10. Relative abundances of fungi at the family level in soils at different time points under fungicide applications in 2020. Time point A: At planting; B: One month post planting. Treatments included Elatus applied at 280.9 ml/A and Non-treated (NT). Taxa with relative abundance < 2% were excluded. Each color represents a different fungal family.

3.4. Discussion

Both Elatus and Aprovia significantly suppressed PED, as well as *V. dahliae* population. Elatus and Aprovia share the same active ingredient, benzovindiflupyr, but Elatus contains second product azoxystrobin. Therefore, Elatus may have a better efficacy over Aprovia and possibly is hard to be overcome by *V. dahliae* developing fungicide resistance (Chapter 2), because the two active ingredients have different modes of action (M. C. Fisher et al., 2018). In addition, the emergence in plots treated with Elatus and Aprovia was significantly higher than in

non-treated plots in 2019. These chemicals might stimulate potato germination but need further investigation.

That lower *V. dahliae* populations in Aprovia-treated plots did not transfer to a higher yield in both years. In contrast, Elatus-treated plots produced 24% and 19% higher yield than Aprovia-treated plots in 2019 and 2020, respectively, although no statistically significant difference was detected. Biological products, Stargus and Regalia also significantly reduced *V. dahliae* populations and mitigated disease symptoms. Numerically, the plots with twice applications of Regalia had the best result. They had advantages in yield increase over Elatus- and Aprovia. Therefore, these products have potential for PED control.

Unexpectedly, none of the treatments showed a statistical difference in yield. The possible reasons might be due to dry weather conditions and large variation. A drought condition may result in a reduction of tuber yield greater than 15% (Deblonde & Ledent, 2001). From May to September of 2020, Maine experienced its worst drought since the 2000s (Birkel, 2020), which may have caused a higher impact on potato yield than disease since the potatoes were not irrigated.

Broad-spectrum fungicides impact soil microbial communities (Wang et al., 2020; Zhang et al., 2021). Families Geobacteraceae, Nocardioidaceae, Sphingobacteriaceae, Streptomycetaceae, and Xanthomonadaceae all increased in all tested plots in both years. In contrast, it was not observed that any of the families showed a consistent increasing or decreasing trend in both years in Elatus-treated plots. In addition, it was observed that the changes in abundance of the fungal families differed significantly between 2019 and 2020. Seventeen fungal families changed in their abundance in 2019 while only five changed in 2020. So this could be the reason for Elatus-treated plots having higher bacterial richness than NT plots in 2019 but lower in 2020. It makes sense that fungicides like Elatus did not affect bacterial

communities, and the community changes were more due to the result of the environment factors, plant growth, and changes in fungal communities.

Fungal composition of the two years differed greatly. There were 23 and 28 families of fungi observed in 2019 and 2020, respectively. Only 11 families were same for both years. This might reflect the variation field location. Aspergillaceae remained the same level. Although some species in Aspergillaceae could be beneficial to disease suppression (Abdallah et al., 2015), changes in its population might be caused by environmental factors as it is not directly dependent on potato. The other 10 families did not show a consistent increasing or decreasing trend in both years. Most of them appear to be neutral regarding their interactions with plants, they participate in nutrient cycling but do not directly affect disease on plants. For example, Mortierellaceae was the most represented family in both years, and it was reported to be beneficial by contributing to the phosphorus cycle in the soil (F. Li et al., 2018; Loit et al., 2020). Trichocomaceae and Corticiaceae were two families that might be beneficial to potato growth. *Penicillium oxalicum* in Trichocomaceae could suppress the disease caused by nematodes (Martinez-Beringola et al., 2013) while the microorganisms in Corticiaceae could have the potential to control the disease caused by *Rhizoctonia solani* and *Pythium* spp. (Burdsall Jr et al., 1980).

In Elatus-treated plots, 7 families of fungi increased and 10 families decreased in abundance in 2019, and 3 families increased and 2 decreased in abundance in 2020. None of them had a consistent trending of change in both years. Therefore, it is difficult to conclude which neutral or beneficial fungal families are promoted or suppressed under the application of Elatus. However, fungal richness and diversity were higher in Elatus-treated plots than in NT plots (except Shannon index in 2020). In addition, the population of *V. dahliae* decreased under Elatus application, which supported the disease evaluation data.

3.5. Conclusions

Elatus and Aprovia significantly reduced PED. Stargus and Regalia also showed significant inhibition of PED with a similar effect. Soil microbial community structure and diversity were affected after Elatus application. Neutral or beneficial fungi rebounded back more quickly after soil treatment compared to pathogen.

CHAPTER 4

EFFECTS OF SOIL FUMIGATION ON POTATO EARLY DYING AND SOIL MICROBIOME

Chapter Abstract

Verticillium dahliae is a soilborne pathogen causing potato early dying (PED). Soil fumigation has been widely used to reduce its population. In this study, a two-year field trial using soil fumigation with Vapam (a.i. metam sodium) at 0, 35, 45 and 50 gal/A was conducted at Aroostook Farm, Presque Isle, ME. Oat-seed-mediated inoculum of *V. dahliae* was evenly incorporated in soil prior to fumigation in the fall. Potato 'Russet Burbank' was planted in the following Spring. PED was evaluated since the first symptoms was observed in the growing season. Tuber diseases and yield were measured after harvest. Genomic DNA was extracted from soil samples collected at two weeks after fumigation, at planting, two months after planting and two weeks before harvesting for genetic analysis. *Verticillium dahliae* in soil was measured using quantitative polymerase chain reaction (qPCR). Illumina MiSeq was used for sequencing the V4 region of the 16S rRNA gene of bacteria and ITS1 region of fungi. Operational taxonomic unit (OTU) was used to analyze the sequence and microbial taxa. Fumigation at all three rates decreased incidences of plant disease by 48% to 67% and reduced tuber diseases by 51% to 67%, but no significant differences were observed with increasing dosage. Vapam decreased *V. dahliae* populations in soil by up to 89%. In soil microbiome at the family level, diversity, richness, and abundance were affected by fumigation. Vapam at 50 gal/A had a similar effect on bacterial communities as 35 gal/A, and a greater impact on fungal communities than 35 gal/A. After fumigation, most bacterial and fungal families that recovered rapidly were non-pathogenic. Thus, Vapam was effective in reducing *V. dahliae*, but its impact on soil microbiome needs to be addressed.

4.1. Introduction

Potato early dying (PED), caused by *Verticillium* spp. with *V. dahliae* being the predominant species, is a yield-constraining disease (Martin et al., 1982; Pegg & Brady, 2002). Potato yield may be decreased by up to 50% by its infection (K Johnson et al., 1986; Powelson & Rowe, 1993). *Verticillium dahliae* can survive in soil for over 14 years (Wilhelm, 1955) and has more than 200 species of hosts (Berlanger & Powelson, 2005; Klosterman et al., 2009), which make the disease extremely hard to control. Currently, the most effective strategy for controlling PED is reducing pathogen populations in soil (Li et al., 2017), and soil fumigation has been used for decades for this purpose (Giovanni Bubici et al., 2019).

The most frequently used fumigant for PED control is metam sodium (sodium N-methyldithiocarbamate). Vapam is a common trade name of this product (AMVAC, Commerce, CA, United States). It takes effect by decomposing into several derived compounds, with methyl isothiocyanate (MITC) as the major active ingredient, which is more toxic than the original structure to suffocate organisms, particularly pathogens, pests, and weeds (Zheng et al., 2006). It is noticeable that due to its broad-spectrum toxicity, the whole soil microbiome is impacted (J. Li et al., 2017; Sederholm et al., 2018).

A soil microbiome includes bacteria, fungi, and archaea, which can be measured by microbial abundance and structure (Larkin et al., 2011; Stark et al., 2007). Some of them are plant pathogens, but some are beneficial. Many microorganisms can inhibit the growth of pathogen by secreting anti-biotic metabolites or by parasitizing pathogens (Dicklow & Madeiras, 2018; Nihorimbere et al., 2011). They can also indirectly affect diseases by inducing plant defense (Prasad et al., 2015) or fixing nutrients (Roy & Singh, 1994). Some microorganisms may not be antagonistic but suppress pathogens by competing for space and nutrients (Abdullah et al., 2017). Our goal is expected to reduce pathogen populations while maintaining beneficial microorganisms.

This study aimed to evaluate the impact of soil fumigation by Vapam on potato yield, PED, *V. dahliae* populations, changes in soil bacterial and fungal communities, and determining optimal rate of application. Therefore, the result may contribute to sustaining and increasing the benefit of both the potato yield and potato business in Maine and other potato production areas.

4.2. Materials and Methods

4.2.1. *Verticillium dahliae* isolates and inoculum preparation

Verticillium dahliae isolates were obtained from diseased potato stems in Maine potato fields. Genomic DNA of *V. dahliae* isolates was extracted and used as a template for performing polymerase chain reaction (PCR) targeting the ITS gene. PCR products were sequenced and analyzed using basic local alignment search tool (BLAST) algorithm against the National Center for Biotechnology Information (NCBI) database for positive identification.

Inoculum of *V. dahliae* was prepared using double-sterilized oat seed as a medium. Ten plugs of freshly cultured *V. dahliae* isolates were transferred into a mushroom growing bag having an air filter that contained 6 liters of oat seed, which was autoclaved for 45 min twice in 24 hr. The bag was incubated at 22 ± 1 °C for at least four weeks. During incubation, the bags were shaken every other day for better inoculum distribution and aeration. After the incubation, the inoculated oat seed was air dried and stored at 4 °C until use.

4.2.2. Field trials

A field trial was established at the Aroostook Research Farm, Presque Isle, Maine, in 2019 to 2020. A 2-row plot with 25-ft-long beds was arranged in September 2019 as a randomized complete block design (RCBD) with 4 replications. *Verticillium dahliae* inoculum was evenly spread on soil surface at the rate of 20 ml/foot and lightly incorporated in the soil with a rototiller on September 16th,2019. For control purposes, one plot was not infested with *V. dahliae* and used as a negative control. Vapam (a.i. 42.0% metam sodium) was applied at 35, 45, and 50 gallons per acer on inoculated plots with a fumigant applicator on September 19th, 2019.

Non-fumigated plots with or without *V. dahliae* infestation were included for control, Potato 'Russet Burbank' seed pieces were planted on May 27th, 2020, using a planter at a plant spacing of 16 inches and a row spacing of 3 feet. Fertilizer (N:P:K = 14:14:14) was applied at planting at 1,400 lb/A. The plots were maintained using local standard operations.

Plant emergence was observed on June 26th, 2020. Disease symptoms were evaluated on September 5th. Disease incidence (%) was calculated as (number of symptomatic plants / number of total plants) x 100%. Potato vines were killed on September 10th. Harvest was done on September 24th. Potato yield was determined by weight, and tuber disease was measured by examining 50 tubers that were cut into two parts. Tuber disease incidence was calculated as (number of diseased tubers / number of total tubers) x 100%.

Soil samples were taken by compositing 15 samples per plot collected at random positions in the furrow by a 6-inch hand trowel. Sampling time points included post fumigation (October 8th, 2019), at planting (May 27th, 2020), two months after planting (mid-season) (July 29th, 2020), and before harvest (September 5th, 2020). Soil samples were immediately put in an iced cooler and transported to the lab. The soil was sieved through a 10-mesh (2 mm) sieve to remove the rocks and weeds, then stored in 15 ml centrifuge tubes in a -80°C freezer.

4.2.3. Soil DNA extraction

Genomic DNA was extracted from 0.25 g sampled soil per plot using the DNeasy PowerSoil Pro Kit (QIAGEN Inc., Germantown, MD, United States) following the manufacturer's instructions. DNA concentration was quantified with a NanoDrop 2000 (Thermo Fisher Scientific, Wilminton, DE, United States). Soil DNAs were stored in a -80°C freezer until use.

4.2.4. Quantification of *Verticillium dahliae*

Quantitative polymerase chain reaction (qPCR) was performed using *V. dahliae*-specific primers VertBT F and VertBT R (Atallah et al., 2007). Reaction mix was prepared by adding 1

µl DNA, 10 µl Luna® Universal qPCR Master Mix (New England Biolabs Inc., Ipswich, MA, United States), 0.5 µl forward primer (VertBT_F 5′-AACAACAGTCCGATGGATAATTC3′) and reverse primer (VertBT_R 5′-GTACCGGGCTCGAGATCG-3′), and adjusted to 20 µl with DNase-free water. Prepared reaction mixes were loaded on Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States). Thermal cycler settings were 95℃ for 3 min, followed by 40 cycles of 95℃ for 10 sec and 63℃ for 35 sec (Aljawasim & Vincelli, 2015). A standard curve was established by amplifying pure *V. dahliae* DNA at 20 ng/ul, 2 ng/ul, 0.2 ng/ul 0.02 ng/ul and 0.002 ng/ul and used for calculating DNA concentration of *V. dahliae* in soil samples.

4.2.5. Soil microbiome analysis

4.2.5.1. Bacterial community

Primer pair 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACHVGGGTWTCTAAT) was used to amplify 16S rRNA gene V4 region (Kozich, 2013). PCR mix was prepared by adding 5 µl of 5x Green GoTaq reaction buffer, 0.5 µl of dNTPs, 0.5 µl of each pair of primers, 0.13 µl of GoTaq DNA Polymerase, 1 µl of DNA, and adjusted to 25 µl using DNase-free water. Thermal cycler setting was 3 min at 95°C, followed by 30 cycles of 45 sec at 95°C, 60 sec at 50°C and 90 sec at 72°C, with a final elongation of 10 min at 72°C. PCR amplicons were examined by gel electrophoresis (2%) to ensure they were the expected size. DNA from Vapam at 35 gal/A and 50 gal/A fumigated plots were diluted into 4 ng/ul and submitted to Michigan State University Genomic Core Facility (East Lansing, MI, United States) for Illumina sequencing. Submitted DNA was amplified by same Illumina compatible amplicon libraries of the 16S rRNA V4 hypervariable region using barcoded primer pair 515f/806r. The sequencing was performed via Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) using a 2 x 250 bp paired end format.

4.2.5.2. Fungal community

Two-step PCR was used for fungal community sequencing. The first step was to amplify the soil DNA samples using ITS1/ITS2 primer pair with tags on the 5' ends (CS1-ITS1: ACACTGACGACATGGTTCTACA – TCCGTAGGTGAACCTGCGG; CS2-ITS2: TACGGTAGCAGAGACTTGGTCT - GCTGCGTTCTTCATCGATGC). DNA from Vapam at 35 gal/A and 50 gal/A fumigated plots were selected for the amplification. Reaction mix was prepared in a 25 µl volume using the same formulation as bacterial amplification. Thermal cycler setting was 2 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 53°C and 60 sec at 72°C, with a final elongation of 10 min at 72°C. A gel electrophoresis (2%) was used to confirm the amplicons were at the expected size, and off-target products were not present. PCR amplicons were diluted into 4 ng/ul and submitted to Michigan State University Genomic Core Facility (East Lansing, MI, United States) for Illumina sequencing. Submitted samples were then amplified by Illumina compatible amplicon tags using barcoded primer pair CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT). The sequencing was performed via Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) using a 2 x 250 bp paired end format.

4.2.6. Data analysis

Data were analyzed using SAS studio (SAS Institute, Cary, NC, United States). General linear model (GLM) was used for examining treatment effects. The Student-Newman-Keul test ($\alpha = 0.05$) and Fisher's LSD test ($\alpha = 0.05$) were used for the analysis of emergence rate, disease incidences, and yield. The Illumina sequencing data were analyzed using the MOTHUR software pack (version 1.39.5, https://mothur.org/). Operational taxonomic unit (OTU) was used to group microbes based on 97% similarity (Schloss et al., 2009). Sequences were processed according to the MiSeq SOP (https://mothur.org/wiki/miseq_sop/), including reducing sequences and PCR errors, finding unique sequences, aligning sequences to the Silva reference database for bacteria

and to the UNITE reference database for fungi (UNITE, 2019; Yilmaz et al., 2014), and assessing error rates. Visualization of the outputs was processed in RStudio (http://www.rstudio.com/). Relative abundance at 0.5% was used as a cut-off point for family-level bacterial community analysis, and 2% was used for fungal community analysis.

4.3. Results

4.3.1. Soil fumigation effects on PED and potato yield

Fumigated plots at different rates of Vapam application had a similar effect, reducing the incidences of plant disease by 48% to 67% and tuber diseases by 51% to 67% (Figure 4-1). All Vapam-treated plots had significantly lower PED incidences than the NT plots. Tuber disease incidences were also lowered by Vapam applications, but only 45 gal/A were statistically different from non-treated plots (Figure 4-1). Disease incidences did not decrease with increasing Vapam dosage. Instead, the lowest point was found in the 45 gal/A plots. The 35 gal/A and 50 gal/A plots were similar in terms of disease incidence (Figure 4-1). However, no differences in yield were observed under Vapam application at different rates (Table 4-1).

Table 4-1. Effects of soil fumigation with Vapam on potato yield

Treatment	Soil infestation [z]	Yield (cwt/A)
Vapam (@ 35 gal/A)	Yes	157.32 a [y]
Vapam (@ 45 gal/A)	Yes	148.80 a
Vapam (@ 50 gal/A)	Yes	152.34 a
Non-treated	Yes	164.39 a
Non-treated	No	184.00 a

[z] *Verticillium dahliae* inoculum grown in oat seed was applied at 20 g/foot. [y] Means of yield followed by the same letter are not significantly different at $\alpha = 0.05$ as determined by Fisher's LSD test.

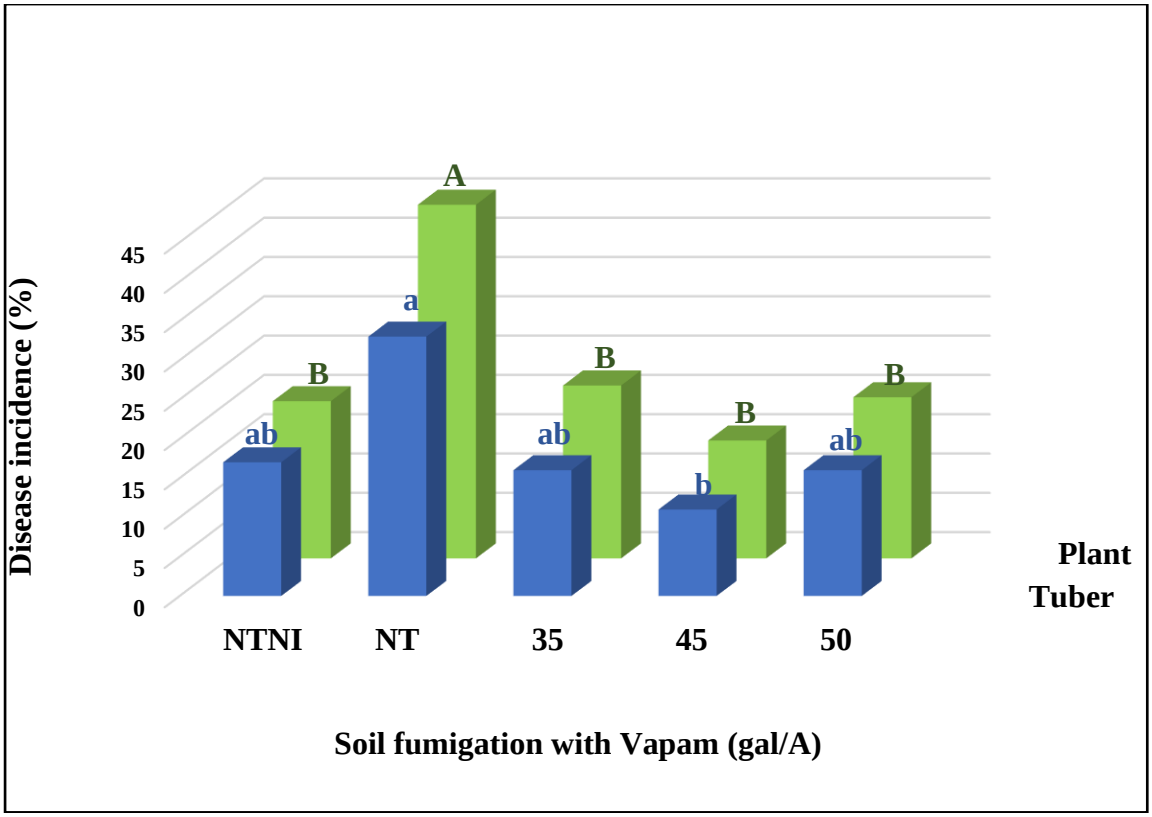

Figure 4-1. Plant and tuber disease incidences in a field trial. Treatments included non-treated and non-inoculated (NTNI), non-treated (NT), and Vapam at 35 gal/A, 45 gal/A, and 50 gal/A. Significance was determined by Student-Newman-Keuls test ($\alpha = 0.05$).

4.3.2. *Verticillium dahliae* quantification

qPCR results revealed that Vapam fumigation at all three rates decreased *V. dahliae* populations (Figure 4-2). Vapam at 45 gal/A had an overall consistent efficacy and was the closest to the NTNI among all three different dosages. Plots applied with 50 gal/A Vapam had best performance at planting and at mid-season, *V. dahliae* inoculum decreased by 89% and 60%, respectively, but an increase in *V. dahliae* was observed before harvest (Figure 4-2).

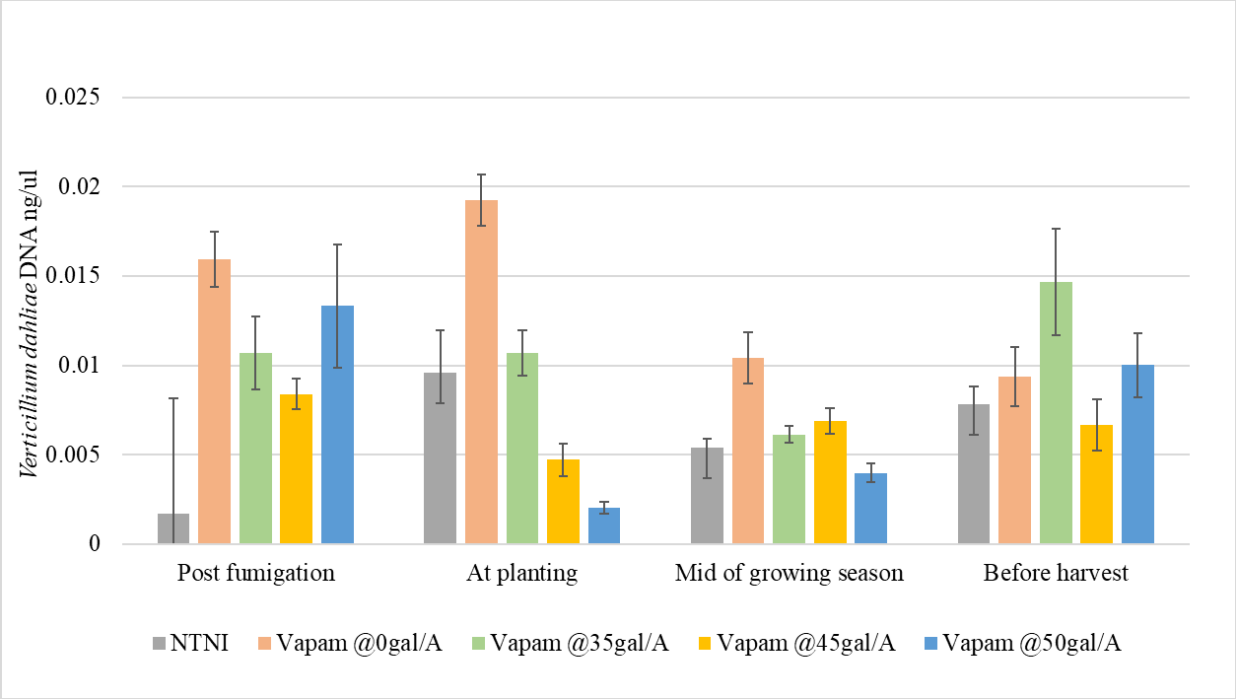

Figure 4-2. Quantitative estimation of *Verticillium dahliae* in soils treated with Vapam at 0, 35, 45, and 50 gal/A, and at time points analyzed by quantitative polymerase chain reaction. NTNI: non-inoculated and non-treated used for control. Error bar was used to determine significance.

4.3.3. Bacterial community changes under Vapam fumigation

A total number of 3,584,853 reads and 36,647 OTUs were obtained. Observed OTUs, Chao1, and ACE indices indicated that highest bacterial richness was observed in the NT plots two weeks post fumigation. Comparison among time points showed that the lowest relative abundance occurred at planting. The NT plots exhibited overall higher richness than Vapam fumigated plots at the time points of post fumigation, at planting, and before harvest. Richness in fumigated plots increased to a similar level as the NT plots at mid-season (Figure 4-3). Shannon diversity index showed the same trend. Vapam fumigated plots resulted in a lower diversity of bacteria than NT plots two weeks after fumigation and before harvest. For all treatments, the highest diversity was observed at two weeks after fumigation, with lower diversity for all treatments at subsequent time points (Figure 4-3).

Forty-two families were classified in the bacterial communities (Figure 4-4), and all bacteria that could not be classified to family taxonomic level were grouped as "Other". Excluding “Other”, the most abundant families were Sphingomonadaceae (7.04%), Micrococcaceae (4.36%), and Conexibacteraceae (2.68%). Among them, Vapam did not result in significant changes in abundance of Sphingomonadaceae, and the abundance peaked at mid-season. Micrococcaceae abundance in the NT plots was lower than in the fumigated plots at all time points, with the greatest difference observed at the post fumigation sampling. Conexibacteraceae abundance was higher in the 50 gal/A Vapam fumigated plots than the NT plots at all time points, same pattern was observed for Intrasporangiaceae. In contrast, the abundance of Planctomycetaceae was lower in the 50 gal/A Vapam fumigated plots than in the NT plots. The highest abundance was observed at the time point of post fumigation. The abundance of Bacillaceae decreased in all fumigated plots and remained slightly lower than that in the NT plots (Figure 4-4). Moreover, Phyllobacteriaceae could not be observed after fumigation, but it was observed at planting and at mid-season and was more abundant in fumigated plots than in NT plots. Abundance of Microbacteriaceae in the NT plots was lower than in the fumigated plots at two weeks post fumigation, and it increased to higher than the fumigated plots at planting but decreased to less than the fumigated plots at subsequent timepoints. Polyangiaceae abundance in the fumigated plots decreased at two weeks post fumigation, and reduced to non-detectable at subsequent timepoints (Figure 4-4).

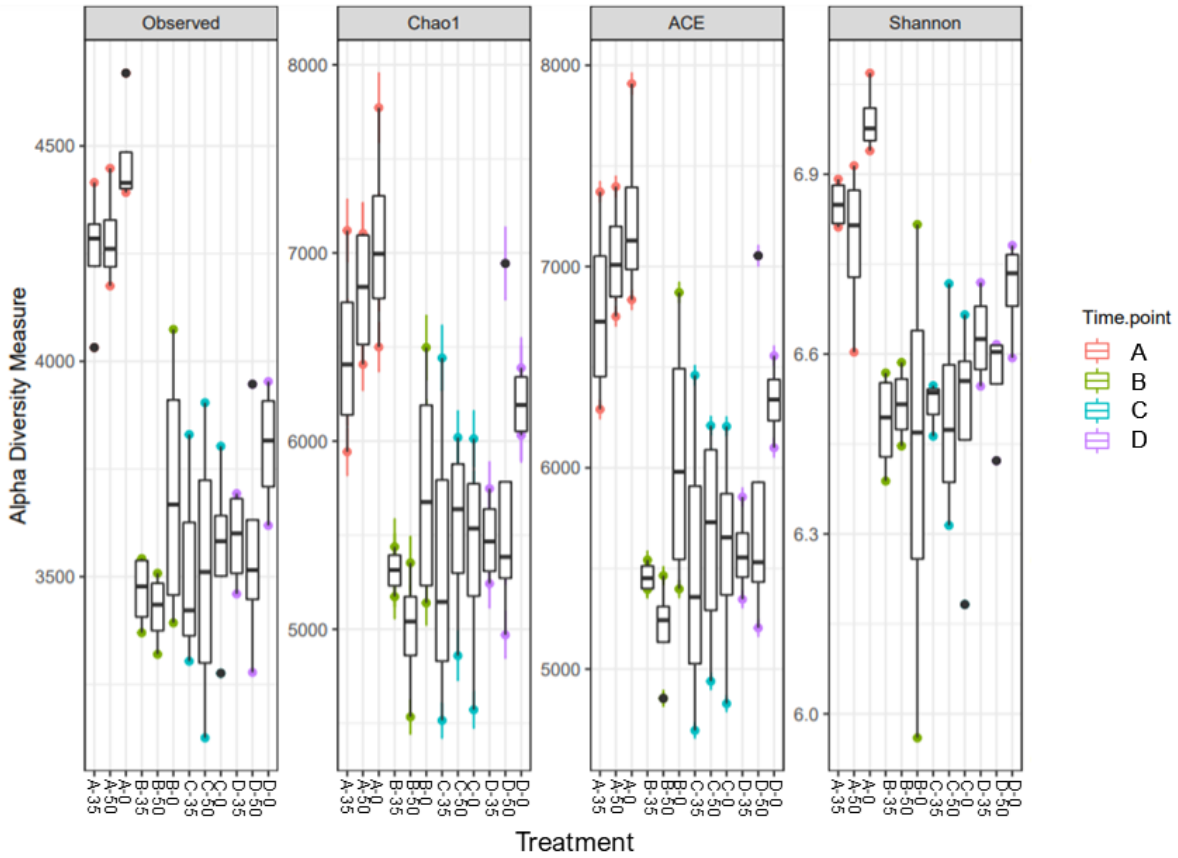

Figure 4-3. Bacterial alpha diversity in soils at different time points after fumigation: A: two weeks post fumigation; B: at planting; C: two months after planting (mid-season); D: one week before harvest. Observed: box plot of observed operational taxonomic unit (OTU). Chao1: box plot of bacterial Chao1 index. ACE: box plot of bacterial ACE index. Shannon: box plot of Shannon diversity index. Treatments included 0, 35 and 50 gal/A Vapam applied.

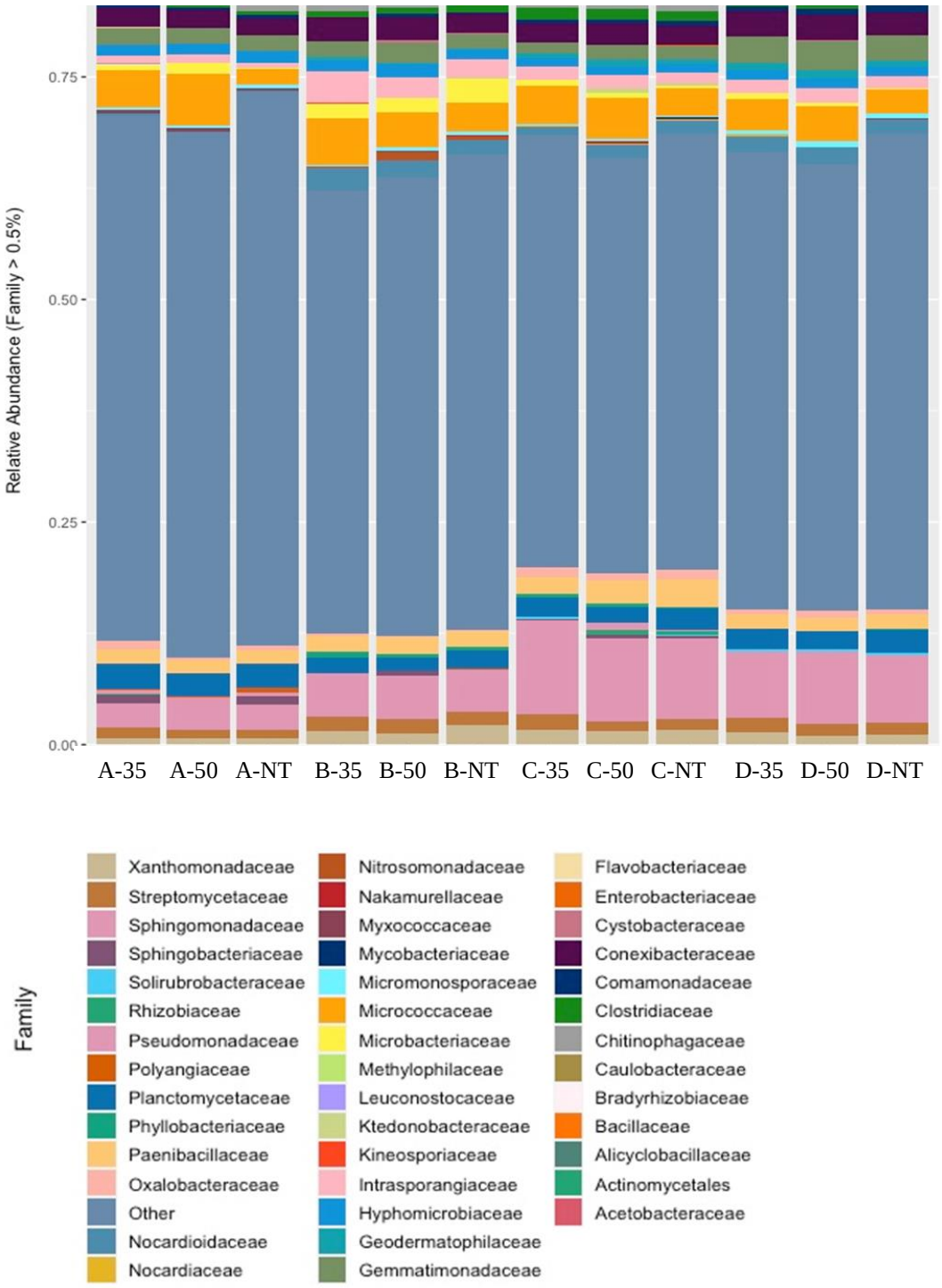

Figure 4-4. Relative abundances of bacteria at the family level in soils at different time points after fumigation: A: two weeks post fumigation; B: at planting; C: two months after planting (mid-season); D: one week before harvest. Treatments included 0, 35 and 50 gal/A Vapam applied. Taxa with relative abundance < 0.5% were excluded. Each color represents a different bacterial family.

4.3.4. Fungal community changes under different Vapam dosage

A total number of 3,102,919 reads and 17,536 OTUs were obtained. Observed OTUs, Chao1, and ACE richness estimators revealed that although a reduction in richness was not observed two weeks post fumigation, the effects of fumigation on the fungal communities were evident at planting and mid-season (Figure 4-5). According to Shannon diversity index, the highest diversity was observed in 50 gal/A Vapam fumigated plots two weeks post planting, while the lowest diversity was in 35 gal/A Vapam fumigated plots. Diversity in 50 gal/A Vapam fumigated plots and the NT plots dropped at planting, but they increased at mid-season. All plots reached a similar diversity before harvest, with diversity in the NT plots being relatively higher (Figure 4-5). Overall, fungal diversity and abundance increased with potato growth and decreased when the field was vacant.

Nineteen fungal families were classified as shown in Figure 4-6. All fungi that could not be classified were grouped as "Other". The most abundant families were Aspergillaceae (30.37%), Trichocomaceae (22.09%), and Mortierellaceae (19.59%). Abundance of Aspergillaceae was higher in the fumigated plots than in the NT plots two weeks post fumigation and was lower than that of the NT plots at subsequent time points. Abundance of Trichocomaceae was higher in the fumigated plots than in the NT plots for all timepoints. Fumigation resulted in a lower abundance for Mortierellaceae two weeks post fumigation, however, the abundance increased at subsequent timepoints. In addition, Mrakiaceae abundance in the fumigated plots was lower than in the NT plots for all timepoints, with the differences being closest two weeks post fumigation. Microdochiaceae was first observed in 50 gal/A Vapam fumigated plots at planting, and the abundance remained higher than 35 gal/A Vapam fumigated plots and the NT plots at subsequent timepoints. Rhizopodaceae abundance in the fumigated plots was undetectable until mid-season, and it dropped to undetectable level before harvest. Didymellaceae and Bulleribasidiaceae could not be detected two weeks post fumigation.

However, Didymellaceae was found at mid-season, and the abundance was higher in the fumigated plots than that of the NT plots; Bulleribasidiaceae was not detected until harvest, and the abundance was higher in the fumigated plots than that of the NT plots. Myxotrichaceae abundance was higher in the fumigated plots than that of the NT plots at planting and mid-season, but they dropped to undetectable level at subsequent time points (Figure 4-6).

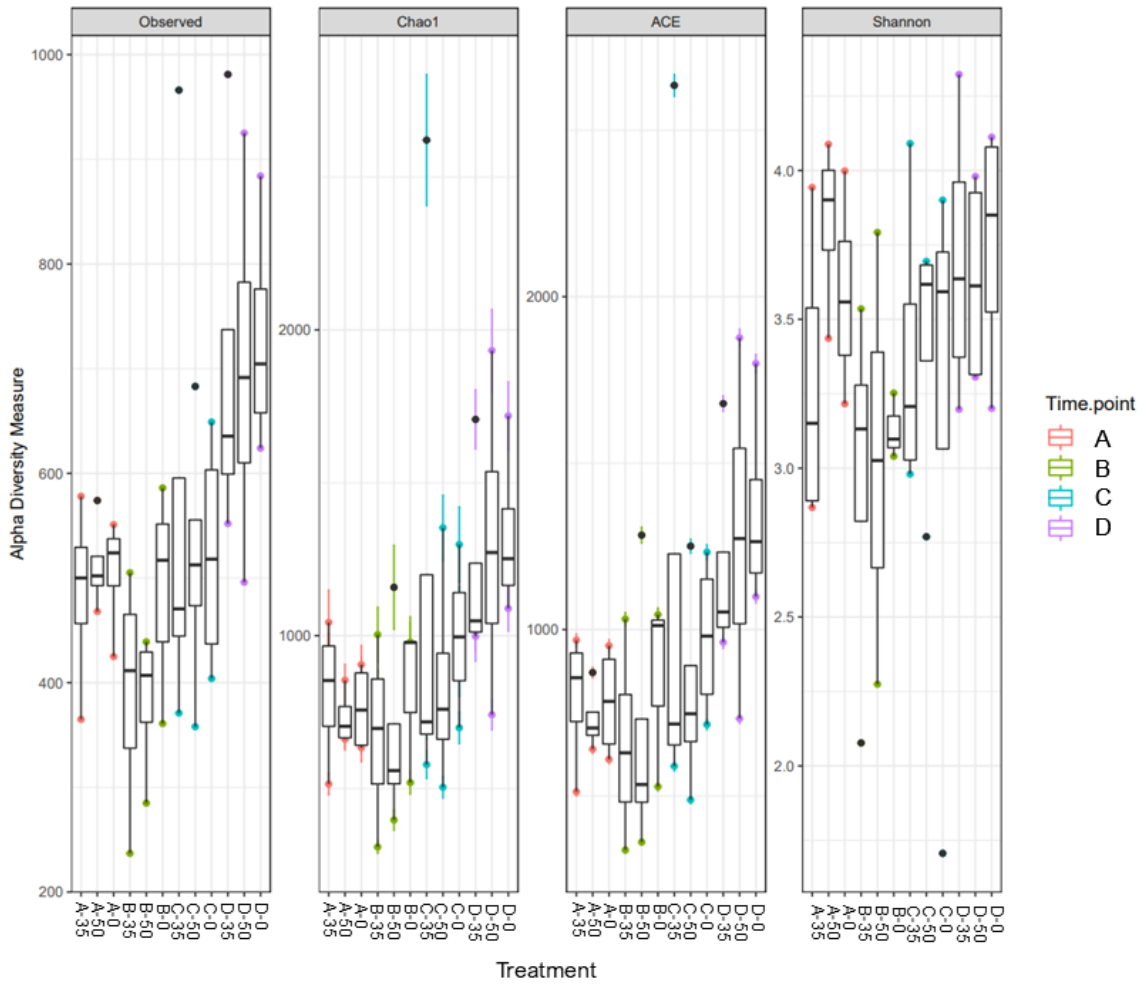

Figure 4-5. Fungal alpha diversity in soils at different time points after fumigation: A: two weeks post fumigation; B: at planting; C: two months after planting (mid-season); D: one week before harvest. Observed: box plot of observed operational taxonomic unit (OTU). Chao1: box plot of bacterial Chao1 index. ACE: box plot of bacterial ACE index. Shannon: box plot of Shannon diversity index. Treatments included 0, 35 and 50 gal/A Vapam applied.

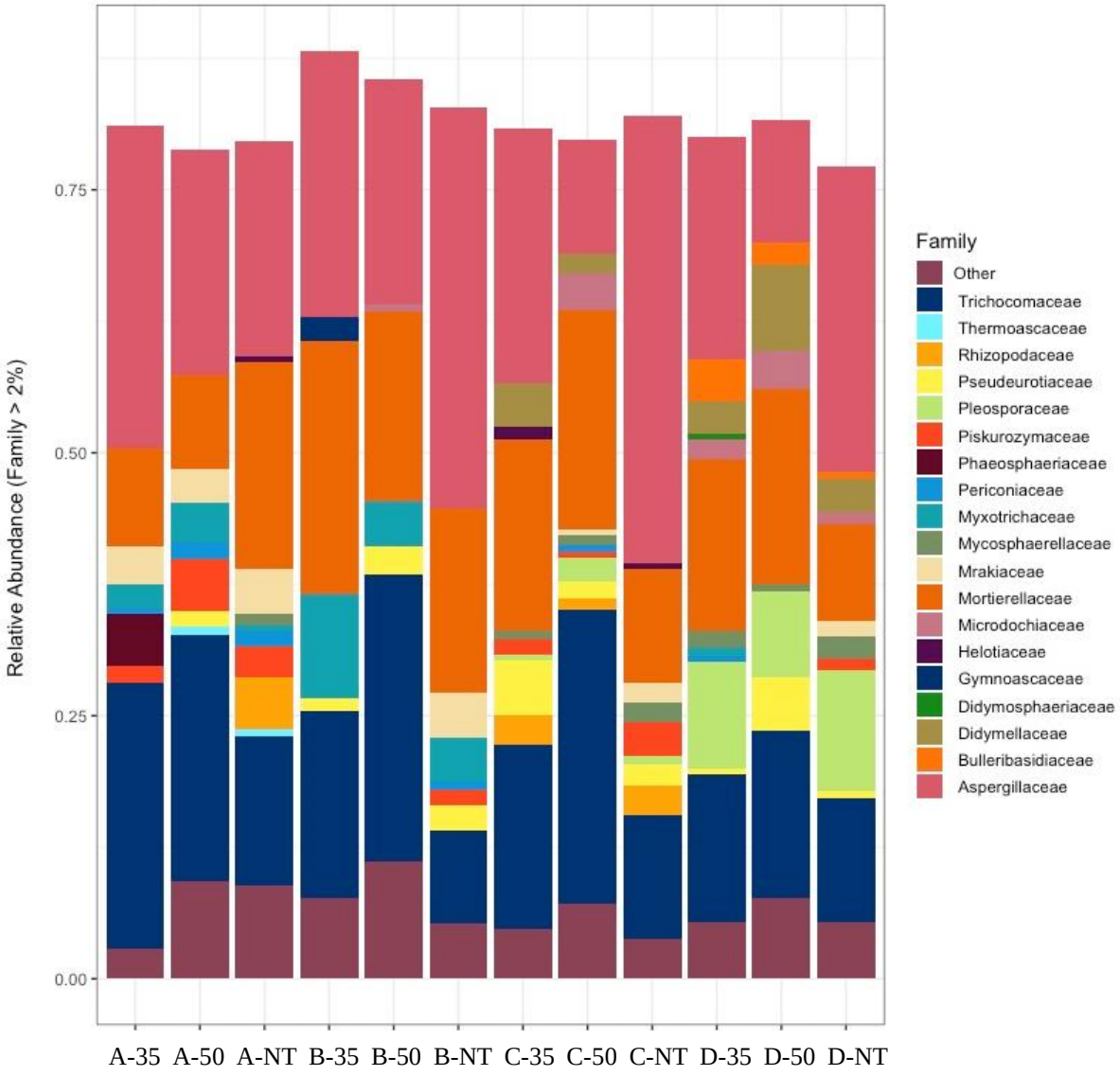

Figure 4-6. Relative abundance of fungi at the family levels in soils at time points after fumigation with Vapam at 0, 35, and 50 gal/A: A: two weeks post fumigation; B: at planting; C: two months after planting (mid-season); D: one week before harvest. Taxa with relative abundance < 2% were excluded. Each color represents a different fungal family.

4.4. Discussion

Soil fumigation using metam sodium has been shown to effectively suppress the population of *V. dahliae* and PED as well as increase potato yield in many studies (Pasche et al., 2013; Taylor et al., 2005; L. Tsror et al., 2005; Yellareddygari & Gudmestad, 2018). In some cases, disease is reduced but no yield benefit is obtained under soil fumigation (Neilson et al.,

2020). In this study, soil fumigation reduced PED and *V. dahliae* population in soil, but effects on yield were not significant. Part of the reason for this was possibly due to weather conditions (Deblonde & Ledent, 2001), as from May to September of 2020, Maine experienced its worst drought since the 2000s (Birkel, 2020).

That yield was not promoted by fumigation can be explained in several ways in this study. Firstly, field disease incidences on both stems and tubers were lowered by soil fumigation, which was supported by qPCR data. After fumigation, *V. dahliae* populations in both 45 gal/A and 50 gal/A plots decreased substantially and remained low until planting. This was most obvious at planting, where an application rate of ≥ 45 gal/A reduced *V. dahliae* populations to even lower than non-inoculated plots. This may be due to the pathogen needing the host plant to effectively multiply. To maximize the interval between fumigation and planting will help maximize competition between pathogens and neutral microorganisms (Abdullah et al., 2017).

Unexpectedly, 50 gal/A Vapam application resulted in an increase in *V. dahliae* populations near the end of the season. This could be explained in part by the slow rebound of the probiotic microbes after being eliminated by fumigant, and therefore the suppression to *V. dahliae* or plant protection reduced. qPCR data also indicated that Vapam at 45 gal/A provided a more consistent efficacy as the *V. dahliae* population in 45 gal/A Vapam fumigated plots was lower than in the NT plots at all timepoints. Although this is not as expected, the fact was that increasing the fumigant dosage may not always lead to better performance. Similar results can be found in other studies, for example, Pasche et al. (2014) found that metam sodium at 467 l/ha (comparable to 50 gal/A) resulted in lower *V. dahliae* population than at 373 l/ha, 561 l/ha, 655 l/ha. Similar studies also revealed that different dosages of metam sodium at different injection depth resulted in different effects, and the highest dosage did not always have the best efficacy (Yellareddygari & Gudmestad, 2018). Higher dosages of metam sodium might benefit the yield by eliminating more pathogen, while lower dosage might benefit the yield by lowering the

impact of fumigant to beneficial microorganisms. Therefore, balancing the suppression of pathogens and reducing the impact of fumigant on the soil microbial communities may be the key to find the optimal dosage. The results in this study suggested Vapam at 45 gal/A might be the sweet spot that balanced the impacts on the beneficial and pathogenic microorganisms.

Soil fumigation with metam sodium impacts the diversity and structure of the soil microbial community (Collins et al., 2006; J. Li et al., 2017; Macalady et al., 1998; Toyota et al., 1999). In this study, alpha diversities of both bacterial and fungal communities were impacted by fumigation. For bacterial communities, the highest richness and diversity occurred in the NT plots two weeks post fumigation, and the richness/diversity advantage in the NT plots was retained all the way through to harvest. This indicated that Vapam affected bacterial communities, and the richness and diversity of bacterial community in fumigated plots did not quickly recover. In addition, bacterial richness and diversity decreased between fumigation and at planting. This might be affected by the cold weather during the winter. Most of the bacteria that increased in fumigated plots are considered commensals to plants, such as bacteria in Conexibacteraceae, Intrasporangiaceae, Micrococcaceae. In addition, some families of potentially beneficial bacteria such as Bacillaceae decreased after fumigation (Gomaa, 2012; B. Li et al., 2013; S. Li et al., 2013). This might help to explain why higher dosages did not lead to higher yield.

An interesting pattern was observed in fungal communities. Under the treatment with Vapam at 50 gal/A, the highest diversity but relatively low richness occurred post fumigation. This indicated that many fungi were killed by Vapam, resulting in abundant space and nutrients becoming available. As a result, low populations of remaining fungi in diverse groups grew unhindered, and diversity increased. However, the diversity advantage in 50 gal/A Vapam fumigated plots was not retained at subsequent timepoints. This might be due to the less competitive fungi being able to multiply during the window period, but not when other

competitive fungi became predominant. In the fungal communities, families that increased in their population during potato growth were mainly neutral, while some of them contained pathogenic species, such as Trichocomaceae (Stošić et al., 2020). Overall, the effects Vapam at different rates were similar after fumigation, but higher Vapam rate resulted in higher richness eventually. Vapam at a higher rate resulted in higher diversity post fumigation but converged with that in lower Vapam treated plots at later stages.

4.5. Conclusion

Soil fumigation by Vapam significantly reduced the severity and incidence of PED at rates greater than 30 gal/A. Fumigation impacted the diversity, richness, and abundance of both bacterial and fungal communities. After fumigation, pathogens are suppressed but non-pathogenic fungi rapidly recovered.

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