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Chemical and Non-Chemical Control of Potato Pink Rot

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CHEMICAL AND NON-CHEMICAL CONTROL OF POTATO PINK ROT

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CHEMICAL AND NON-CHEMICAL CONTROL

OF POTATO PINK ROT

By Xuemei Zhang

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An Abstract of the Thesis Presented
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Pink rot of potato (*Solanum tuberosum*) is a widespread soilborne disease that causes significant losses in the field and storage. It is caused by *Phytophthora erythroseptica* (Pethybr.), an oomycete pathogen that produces sexual spores that can survive in soil for years. The management of pink rot mainly relies on chemical control. However, the most effective chemical in pink rot control, mefenoxam, is losing its efficacy owing to the development of mefenoxam resistance in *P. erythroseptica*. To evaluate alternative fungicides (including chemical and biological fungicides) to mefenoxam in pink rot control, two greenhouse experiments and three field trials were conducted. Crop rotation experiments were performed in the field to investigate the rotation effects of alfalfa, barley-ryegrass, canola, red clover, onion, pumpkin, sweet corn and oats on pink rot of potato. Thirty-four wild-type isolates of *P. erythroseptica* were collected for fungicide sensitivity assay and fungicide-resistant *P. erythroseptica* selection, to predict the resistance risk of fluopicolide, an alternative chemical to mefenoxam. Field trials showed that biologicals including *Bacillus subtilis*

(Serenade Soil, Taegro), *Bacillus amyloliquifaciens* (Double Nickel, MBI-110), and extract of *Reynoutria sachalinensis* (Regalia) did not significantly reduce pink rot severity in the harvested potato tubers. The sole application of fluopicolide, some combinations of chemical fungicides (mefenoxam and oxathiapiprolin) and some combinations of chemical and biological fungicides (oxathiapiprolin/fluopicolide and *Bacillus sp*.) significantly reduced pink rot severity in the presence of mefenoxam-resistant *P. erythroseptica* population. In crop rotation trials, alfalfa, canola and pumpkin significantly increased potato tuber yield. However, the rotation crops had no significant effect on pink rot of potato. The results of fungicide resistance study suggested that the risk of *P. erythroseptica* to develop intermediate resistance to fluopicolide was at a medium level, and that there was a trade-off between fluopicolide resistance and biological fitness in *P. erythroseptica*.

DEDICATION

This thesis is dedicated to my families and my friends, for their endless support and encouragement. I am extremely grateful to my parents Fugui Zhang and Chunying Chen. I grew up with their love in a happy family. Without their guidance and support, I would not have been an optimistic person with an open and curious mind. They helped me see the beauty of nature and science. I could not have accomplished my work without them. At last, a special gratitude is sent to my boyfriend Wanxin Chen, who always stands by me and cheers me up.

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LITERATURE REVIEW

Chapter Abstract

Pink rot of potato (*Solanum tuberosum*) is a widespread soilborne disease that causes tuber losses in the field and storage. It has been a persistent problem in potato production since it was first described in 1913. Potato pink rot is caused by *Phytophthora erythroseptica* (Pethybr.), an oomycete pathogen. In addition to direct infection damages, the occurrence of pink rot also leaves potato tubers prone to secondary infections caused by bacteria and fungi. Currently, the management of pink rot mainly relies on chemical fungicide applications. Mefenoxam is a systemic chemical that used to be the most effective synthetic fungicide in pink rot control. However, it becomes less effective owing to the development of mefenoxam resistance in *P. erythroseptica* population. Therefore, new fungicides and management strategies are needed to control pink rot of potato. In recent years, many studies have been conducted using new synthetic fungicides, biological fungicides and crop rotation. An overview of significant literature on the etiology of potato pink rot, pink rot management, fungicide resistance of *P. erythroseptica*, fungicide sensitivity studies and soil microbial community studies is presented here.

Introduction

Potato pink rot is a ubiquitous soil-borne disease caused by an oomycete pathogen, *Phytophthora erythroseptica* (Cairns and Muskett 1933; Gudmestad et al. 2007; Taylor et al. 2004). Pink rot was first described in Ireland (Pethybridge 1913), and then reported in North and South America, Europe, the Middle and Far East and Australia (Rowe and Nielsen 1981). It causes significant losses in the field and storage (Rai 1979; Toms 1968; Yellareddygari et al. 2016). Records from the 20th century showed that in the United States, pink rot caused 10 to 75% tuber loss in the field (Salas et al. 2003). The occurrence of pink rot also leaves potato tubers prone to secondary infections or colonization by bacteria and fungi (O'Sullivan and Dowley 1998).

Pink rot symptom is characterized by the pink or salmon coloration of diseased tubers (Grisham et al. 1983), which is the result of the oxidation of phenolic compounds in potato tubers (White 1946). Pethybridge described that the cut surface of pink rot tuber turned pink in 20-30 minutes, and eventually turned brown or black. Dr. George H. Pethybridge isolated the pathogen and named it as *P. erythroseptica* Pethybridge (Pethybridge 1913).

The first case of potato pink rot caused by *P. erythroseptica* in United States was reported in Maine (Bonde 1938), followed by the ones in Idaho (Goss 1949), New York (Boothroyd 1951), Delaware (Carroll and Sasser 1974), Colorado, Michigan, Minnesota, Nebraska, North Dakota, Wisconsin and other states (Taylor et al. 2002; Venkataramana et al. 2010; Wharton and Kirk 2009).

Etiology of *Phytophthora erythroseptica*

The causal agent of potato pink rot, *P. erythroseptica* is geographically widespread and has a wide host range. Besides potato (*Solanum tuberosum*), it also infects other plant species such as tomato (*Solanum lycopersicum*), barley (*Hordeum vulgare*), crimson clover (*Trifolium incarnatum*), spinach (*Spinacia oleracea*), wild rice (*Zizania palustris*) and other crops (Erwin and Ribeiro 1996; Gillings and Letham 1989; Peters et al. 2005b; Pratt 1981; Whelan and Loughnane 1969).

Phytophthora erythroseptica is a member of the oomycetes, which are not true fungi (Lamour and Kamoun 2009). They used to be considered as a phylum of Kingdom Fungi before polygenetic analyses revealed that they are closer to heterokont algae (brown algae) than to fungi (Fry and Grünwald 2010; Lamour and Kamoun 2009). The oomycetes, known as water molds, share some characteristics with true fungi. They grow filamentous hyphae that enable them to absorb nutrients from the environment (plant tissue, water, animal waste, etc.) and produce spores for reproduction (Murphy 1918; Pethybridge 1914; Vujičić and Colhoun 1966). However, oomycetes and fungi are substantially different (Gisi and Sierotzki 2015). For example, few oomycetes have septa (cross cell walls) in their hyphae, but most fungi have septa. Moreover, the cell walls of oomycetes contain β-1,3, and β-1,6 glucans, but fungi cell walls contain chitin (the polymer of N-acetyl glucose amine) (Fry and Grünwald 2010). Therefore, *P. erythroseptica* is different from the true fungal pathogens on potatoes.

Phytophthora erythroseptica produces various structures including mycelium (an entity consisting of massive hyphae), oogonium (female organ), antheridium (male organ), oospore (sexual spore), sporangium (asexual fruiting body) and zoospore (asexual spore) (Erwin and Ribeiro 1996; Pethybridge 1914). *Phytophthora erythroseptica* rarely forms haustoria (McKay et al. 1957), which are common structures formed on hyphae by some fungi to facilitate the absorption of nutrients from plant cells (Bushnell 1972). In terms of fertilization, *P. erythroseptica* is homothallic, which means its sexual organs (oogonia and antheridia) can mate with ones from the same isolate and produce selfed population (Abu-El Samen et al. 2005; Murphy 1918). Thick-walled sexual spores, oospores, will be produced after mating (Wharton and Kirk 2009). Another common reproductive structure of *P. erythroseptica* is called a sporangium (Chapman and Vujičić 1965). Sporangia are the asexual fruiting bodies of *P. erythroseptica* (Vujičić and Colhoun 1966; Vujičić et al. 1968), which form multiple swimming zoospores.

Phytophthora erythroseptica is a soilborne pathogen that is capable of infecting healthy potato plants in the field (Al-Mughrabi 2009). The mycelia, sporangia, zoospores and oospores of *P. erythroseptica* can initiate pink rot infection on potatoes (Lonsdale et al. 1980; Wharton and Kirk 2009). The thick-walled sexual spores, oospores, serve as overwintering structures in soil, which can survive in soil for years (Erwin and Ribeiro 1996; Nanayakkara et al. 2010). When host plants are planted and the environmental conditions are right, oospores germinate and grow mycelia, which will colonize on roots and basal

stems of potato. During the growing season, zoospores are produced and released from sporangia, and serve as the secondary inoculum source. It is noted that sporangia can also infect plants directly (Erwin and Ribeiro 1996; Wharton and Kirk 2009). A quorum sensing effect was discovered in zoospore communication of *P. erythroseptica*, and the germination of zoospores seemed to be regulated by a zoospore signaling compound and the root exudates of host plants (Jiang et al. 2014).

In some cases, *P. erythroseptica* attacks above-ground parts of the potato plant and causes leaf spots, petiole blights, stem decay, and wilting (Fry and Grünwald 2010; Goss 1949) in the field. However, *P. erythroseptica* usually targets the underground systems (basal stem, root, stolon and tuber) of potato plants. Zoospores also attack tubers through eyes, lenticels and wounds (Fry and Grünwald 2010; Lambert and Salas 2001; Taylor et al. 2004; True 1914; Vargas and Nielsen 1972; Wharton and Kirk 2009). The plant response triggered by the infection of *P. erythroseptica* stimulates plants to release a large amount of phenolic compounds. When an infected tuber is cut, phenolic compounds in the tuber are oxidized and thus the tuber appears to be pink or salmon color (Johnson and Schaal 1957; White 1946). Infected eyes and lenticels turn black and infected tuber tissue becomes rubbery and watery (Fry and Grünwald 2010; Taylor et al. 2004). The author noticed that severe pink rot results in the bursting of lenticels and thus mycelial coverage on tuber lenticels. At the end of each season, *P. erythroseptica* either enters dormancy within soil in the form of oospores or stays in infected tubers (Wharton and Kirk 2009). The latent

pathogens in harvested tubers can spread further in storage (Salas et al. 2000; Stack et al. 1992; True 1914; Yellareddygari et al. 2016).

The infection of *P. erythroseptica* on potato plants usually occurs in warm and poorly-drained fields (Bonde 1938; Cairns and Muskett 1939; Lennard 1980; Smith Jr and Wilson 1978), and pathogens can be spread through infected (including asymptomatic or latent) tubers in storage (Salas et al. 2000; Stack et al. 1992; True 1914). In storage, root, stolon, tuber eyes and tuber lenticels are the natural entrances of *P. erythroseptica* (Lonsdale et al. 1980; Rich 2013), although potatoes are often infected through mechanical bruises and worm wounds (Cunliffe et al. 1977; Taylor et al. 2008).

Management of Potato Pink Rot

Various pink rot management strategies were designed based on the characteristics of *P. erythroseptica* (Secor and Gudmestad 1999). The common strategies include avoiding disease-conducive fields, using certified seed tubers, planting pink rot resistant varieties, managing fertilizer application, managing irrigation, avoiding abiotic stress, avoiding wounds, regulating storage temperature, applying fungicides and rotating potatoes with other crops.

The selection of planting site is critical in pink rot management. Fields with a recent pink rot history must be avoided, since the sexual spores of *P. erythroseptica* can survive in soil for years (Erwin and Ribeiro 1996; Nanayakkara et al. 2010). It is also necessary to test field soil prior to planting, because the characteristics of the soil, such as soil pH, soil nutrients, soil texture

and soil structure, play important roles in pink rot disease cycle. Low pH increases the colonization and infection of *P. erythroseptica* on potato root (Benson et al. 2009a). The availability of soil nutrients determines plant health and thus influences *P. erythroseptica* infection indirectly. Some nutrients like calcium have impacts on *P. erythroseptica* membrane stability and the mobility of zoospores (Messenger et al. 2000). An increase in available calcium can cause a significant reduction in pink rot disease (Benson et al. 2009b). Soil texture and structure determines infiltration rate, the availability of oxygen, and the activities of other soil microbes, and thus has a significant influence on pink rot disease.

It is recommended to grow certified seed tubers and pink rot resistant potato varieties to reduce the risk of pink rot in the field. The susceptibility of potato tubers to various potato diseases including pink rot can be tested before planting (Bohl et al. 1992; Peters and Sturz 2001). Many researchers have tested the susceptibility of different potato varieties to pink rot (Peters et al. 2004; Salas et al. 2003; Thompson et al. 2007). Although all potato cultivars (cultivated varieties) grown in North America are susceptible to pink rot (Peters and Sturz 2001): highly susceptible cultivars include ‘Red LaSoda’, ‘Russet Norkotah’, ‘Goldrush’, ‘Red Gold’, ‘Warba’, ‘Norland’, and ‘Shepody’, still there are moderately resistant cultivars such as ‘Russet Burbank’, ‘Irish cobbler’, ‘Atlantic’, and ‘Pike’ (Benson 2008; Fitzpatrick-Peabody 2008).

Improper fertilization may worsen the damage caused by pink rot. Soil nitrogen, and the balance between soil nitrogen and soil phosphorus have a significant impact *Phytophthora* pathogens (Halsall et al. 1983; Möller et al.

2006). Fertilizers also influences potato plants and soil microbes, which affects the survival of *P. erythroseptica* in indirect ways.

Irrigation management is vital in pink rot control. *P. erythroseptica* thrives in wet environments, and this is why pink rot usually occurs in poorly-drained fields (Bonde 1938; Wharton and Kirk 2009). Over-irrigation should be avoided to minimize the risk of pink rot. It is also necessary to pay attention to weather forecasts, since rainfall can wash away fungicide residues on or around plants. The application of fungicides right before irrigation or rainfall should be avoided.

Potato tubers need to be harvested and handled carefully. It is recommended to harvest potato tubers before frosting, because frost damage assists *P. erythroseptica* in infecting tubers. Temperature regulation is also helpful: cooling down tuber pulp temperature to 65 F prior to harvest will reduce pink rot (Wharton and Kirk 2009). Additionally, tubers should be harvested carefully to avoid mechanical damages on tubers skins (Gudmestad et al. 2007; Secor and Gudmestad 1999). It is encouraged to separate diseased tubers and healthy tubers at harvest. Diseased tubers and plants should be discarded and destroyed before transferring harvested tubers to storages. It is also desirable to take tuber samples to estimate pink rot incidence and tuber yield prior to storage. A recent study introduced a beta regression model for the prediction of pink rot development in storage (Yellareddygari et al. 2016). In this study, tuber yield, the incidence of pink rot in tubers at harvest, and days after harvest were used as variables, and the results demonstrated that the interaction between pink rot and yield is a significant predictor ($\alpha=0.0001$) of pink rot development (Yellareddygari

et al. 2016). Harvested tubers should be cured at 45 to 50 F at 90% (or higher) relative humidity for wound healing. Reducing tuber pulp temperature in storage to 50°F or lower as quickly as possible, using high airflow and preventing water condensation in the pile are useful in postharvest pink rot control (Wharton and Kirk 2009).

The management of potato pink rot is not limited to the above-mentioned strategies. They are usually helpful but not powerful enough to significantly reduce pink rot. In fact, synthetic (chemical) and biological fungicide control and crop rotation are the most effective and important methods in pink rot management.

Chemical control

Oomycetes and true fungi differ in many respects including cell wall and cell membrane composition, metabolic pathways, and sensitivity to a range of inhibitors (Gisi and Sierotzki 2015; Latijnhouwers et al. 2003). It was noticed that oomycetes are insensitive to some conventional fungicides that have suppressive effects on true fungi (Cohen and Coffey 1986; Gisi and Sierotzki 2015; Hirooka and Ishii 2013). For instance, Azole fungicides are a group of fungicides that target the ergosterol biosynthesis pathway and inhibit the ergosterol biosynthesis of true fungi, but they don't have significant suppressive effects on oomycetes, because oomycetes do not synthesize ergosterol (Griffith et al. 1992). A fungicide market share survey (in 2009) showed that over 50% of commercially available fungicides on the market are effective in oomycete control. Among them, 15.5% are specific oomyceticides, and 41% are broad-

spectrum fungicides that also suppress ascomycetes, basidiomycetes and deuteromycetes (Hirooka and Ishii 2013).

Most oomyceticides (oomycete fungicides) were introduced and reported in the late 20th century (Cohen and Coffey 1986; Gullino et al. 2000; Hirooka and Ishii 2013; Schwinn and Staub 1987). There are 4 major (widely-used) groups of oomyceticides within 16 different chemical groups that are effective in oomycete control: the phenylamides (PAs), quinone outside inhibitors (QoIs), carboxylic acid amides (CAAs), and multisite inhibitors (Fungicide Resistance Action Committee 2016; Gisi and Sierotzki 2015; Hirooka and Ishii 2013). The other groups are the ones with unknown modes of actions (MOAs) such as cymoxanil, quinone inside respiration inhibitors (QiIs), fluopicolide, ethaboxam, fosetyl-aluminum and phosphorous acid (Fungicide Resistance Action Committee 2016), or broad-spectrum fungicides, such as fluazinam (Komyoji et al. 1995), which is used to control potato late blight and downy mildews (Gisi and Sierotzki 2015; Hirooka and Ishii 2013). The application methods of chemical oomyceticides are different. Almost all oomyceticide groups are used for foliar treatments, although PAs, fosetyl-aluminum, CAAs, QoIs, QiIs, and fluopicolide can be used in soil treatments. A few of them (PAs, QoIs, QiIs, cymoxanil, hymexazol etc.) have good performance when they are used for seed treatments. Some oomyceticides such as hymexazol and etridiazole are used exclusively for soil applications (Gisi and Sierotzki 2015).

QoI fungicides such as azoxystrobin, famoxadone, and fenamidone, can inhibit mitochondrial respiration of oomycetes. They act through interrupting

electron transport in cytochrome b (complex III) by binding to the Qo site, which is the ubiquinol oxidizing pocket at the outer side of mitochondrial membranes (Gisi 2002). Some of them can inhibit the zoospore liberation and motility of oomycetes (Gullino et al. 2000).

Cinnamic acid amides, valinamide carbamates, and mandelic acid amides are the subgroups of oomyceticides belonging to CAAs. They are effective in controlling most oomycetes, but they are ineffective in the management of *Pythium* and some oomycetes outside *Peronosporales* (Gisi and Sierotzki 2015). The MOA of CAA fungicides acting against *Phytophthora infestans* was revealed: the inhibition of the incorporation of 14C-labeled glucose into the β-1,4 glucan (cellulose) fraction of cell walls of germinating cystospores (Blum et al. 2010). Hence, it was postulated that cellulose synthase was the primary target enzyme of CAAs (Gisi and Sierotzki 2015).

Phenylamide fungicides (PAs), is a group of synthetic chemical compounds that specifically suppress the growth of oomycete pathogens of plants (Gisi and Ziegler 2003; Schwinn and Staub 1987). They act through inhibiting the polymerization of ribosomal RNA biosynthesis in oomycetes (Fisher and Hayes 1982). They are active and versatile, having long-lasting preventive, curative, and eradicative effects in oomycetes control, due to their systemic (acropetal) translocation ability in many crops (Cohen and Coffey 1986; Edgington 1981; Gisi and Ziegler 2003; Ivic 2010; Müller and Gisi 2012). Seven PA chemicals were introduced between 1977 and 2007: metalaxyl and benalaxyl including their active isomers (mefenoxam = metalaxyl-M and kiralaxyl =

benalaxyl-M, respectively), oxadixyl, furalaxyl, and ofurace (Gisi and Cohen 1996). They are single-site inhibitors, thus are often mixed with multisite fungicides or unrelated single-site inhibitors to broaden the fungitoxicity spectrum and slow down the development of fungicide resistance (Gisi and Sierotzki 2015). PAs have significant suppressive effects on the hyphal growth, haustoria, and sporangia formation of oomycetes (Gisi and Sierotzki 2015; Schwinn and Staub 1987).

Metalaxyl and its isomer mefenoxam have been the most effective chemicals in fungicide control of potato pink rot (Fitzpatrick-Peabody and Lambert 2011; Schwinn and Staub 1987; Sukul and Spiteller 2000; Torres et al. 1985). Mefenoxam/metalaxyl acts through inhibiting the polymerase I complex of rRNA synthesis in oomycetes (Davidse 1995). It used to be the most active and widely-used oomyceticide in pink rot control owing to its high efficacy, rapid uptake, high acropetal systemicity, good persistence in plant tissue as well as the protective and curative effects (Schwinn and Staub 1987).

Biological control

The concept of biological control was proposed decades ago and has been widely accepted (Baker and Cook 1974; Baker 1987; Blakeman and Fokkema 1982; Chet 1987). Pal and Gardener defined biological control as the purposeful utilization of introduced or resident living organisms, other than disease-resistant host plants, to suppress the activities and populations of one or more plant pathogens (Pal and Gardener 2006). Generally, it includes the use of beneficial organisms, their genes, and products, such as metabolites, that reduce

the negative effects of plant pathogens and promote positive responses by the plant (Junaid et al. 2013). In research studies and in the market, most biological control agents derive from bacteria (Emmert and Handelsman 1999; Haas and Keel 2003; Schmiedeknecht et al. 1998), fungi (Adams 1990; Butt and Copping 2000; Howell 2003; Whipps and Lumsden 2001) or plant tissues (Harish et al. 2008; Stephan et al. 2005; Su 2012). Researchers have tried various bacterial and fungal biological control agents to manage a wide range of potato diseases including black scurf and stem canker (Brewer 2003; Tariq et al. 2010), brown rot (Kabeil et al. 2008), common scab (Han et al. 2005; Liu et al. 1995), dry rot (Schisler et al. 1997), black leg (des Essarts et al. 2016), late blight (Shanthiyaa et al. 2013; Stephan et al. 2005) and soft rot(des Essarts et al. 2016) on plants or in storage (Elad et al. 1980; Gachango et al. 2012a; Guchi 2015; Lodhi 2004).

Common bacterial biological control agents include *Agrobacterium* (e.g. *Agrobacterium radiobacter*), *Arthrobacter* (Barrows-Broaddus et al. 1985), *Alcaligenes* (e.g. *Alcaligenes faecalis*) (Yokoyama et al. 2013), *Azotobacter* (e.g. *Azotobacter chroococcum*) (Chauhan et al. 2012), *Bacillus* (e.g. *Bacillus subtilis* and *Bacillus amyloliquefaciens*) (Arguelles-Arias et al. 2009; Bais et al. 2004), *Escherichia coli, Enterobacter, Pseudomonas* (e.g. *Pseudomonas aureofaciens* and *Pseudomonas fluorescence*), *Burkholderia, Rhizobium* and so on (Junaid et al. 2013; Narayanasamy 2013b; Saxena et al. 2000). *Agrobacterium radiobacter* starin 84 was the first bacterial biological agent that has been commercialized as a biological fungicide product (Saxena et al. 2000). It is

manufactured by AgBioChem Inc. and called Galltrol (Junaid et al. 2013). Galltrol has been the most effective product for prevention of crown gall disease since it was released in 1985 (AgBioChem_Inc. 2016). Since then, there has been many big progresses in the commercialization of bacterial biocontrol products (Junaid et al. 2013; Parnell et al. 2016). For example, Serenade soil (Bayer/AgraQuest) is a commercial bioproduct that showed suppressive effects on potato late blight (Olanya and Larkin 2006).

Several groups of fungi such as *Ampelomyces, Aspergillus, Coniothyrium, Cryptococcus, Candida, Fusarium, Gliocladium, Penecillum, Phlebiopsis, Pythium* and *Trichoderma* were developed to be biological control products (Butt et al. 2001; Daami-Remadi et al. 2012; Lo 1998). *Trichoderma harzianum* is the most versatile fungal biocontrol, and it showed suppressive effects on a few potato diseases including late blight (Yao et al. 2016), potato leak (Daami-Remadi et al. 2012), stem canker or black scurf (Arora 2008; Hicks et al. 2014) and pink rot (Etebarian et al. 2000).

The extracts of some plants, known as botanicals, are toxic to pathogens (Gurjar et al. 2012). There are three groups of botanicals: plant extracts, essential oils and gels/ latexes. *Clerodendro* sp. plant extract can reduce downy mildew by 60% on pearl millet (Upadhyay et al. 2001). Cassia oil, mustard oil and cinnamon oil showed suppressive effects on *Phytophthora nicotianae* (Narayanasamy 2013a). Oregano oil was found to be effective in potato late blight control (Olanya and Larkin 2006).

The mechanisms of biological control agents have been well-investigated (Azcón-Aguilar and Barea 1997; Baker 1968; Jamalizadeh et al. 2011; Junaid et al. 2013; Lo 1998; Whipps 2001). Generally, the MOAs of biological controls include antibiosis, competition, mycoparasitism, cell wall degradation and induced resistance, plant growth promotion and rhizosphere colonization (Lo 1998).

It is important to understand the relationships between natural organisms, since plant-microbe interaction and microbial interaction are involved in the mechanisms of biological control agents. There are five types of relationships in plant-microbe interactions and microbial interactions, which are parasitism, mutualism, antagonism, commensalism, neutralism (Pal and Gardener 2006). Parasitism means that one organism parasitizes on the other; therefore, one benefits and the other is harmed. The relationship between pathogens and susceptible plants is parasitism. Pathogens are parasites living on or in plants, absorbing nutrients from plants and destroying the plants. Parasitism also exists between microbes (Adams 1990). For example, *Trichoderma* spp. are known as mycoparasites on various fungi such as *Rhizoctonia solani* and *Botrytis cinerea* (Atanasova et al. 2013; Howell 2003; Lorito et al. 1996). Mutualism is a relationship that provides benefits to both species in the relationship. A typical example of mutualism is the interaction between legume crops and *Rhizobium* (Kiers et al. 2003). *Rhizobium* fixed nitrogen for legumes and legumes provide *Rhizobium* with products of photosynthesis in return. Protocooperation is a special form of mutualistic relationship, where the organisms do not depend

exclusively on each other for survival. Unlike *Rhizobium*, many bacterial biocontrol agents are facultative mutualists involved in protocooperations with crops; they do not depend on any specific host to survive and their efficacies depend upon environmental conditions (Pal and Gardener 2006). Commensalism is a relationship, in which one organism benefits and the other is not affected (neither receiving harm nor benefit). Most plant-associated microbes are commensalistic with host plants; they may cause challenges to pathogens and result in a decrease in pathogen infection or disease severity (Pal and Gardener 2006). An antagonistic relationship between organisms causes harm to one or both. Antagonism is the major MOA of most biocontrol agents. Sometimes, the competition between two organisms is considered as a form of antagonism (Junaid et al. 2013). However, antagonism is often used to describe the relationship where antimicrobial metabolites are involved (antibiosis). Many bacterial biocontrol agents secrete antibiotics. For instance, *Agrobacterium radiobacter* releases Agrocin 84 and *Bacillus* spp. can produce Bacillomycin D, Zwitermycin A, Mycostubilin etc (Junaid et al. 2013). Plants can also secrete antimicrobial compounds and establish an antagonistic relationship with pathogens (Gurjar et al. 2012; Narayanasamy 2013a). Neutralism describes an interaction where organisms do not have effects on each other (Baker 1968; Pal and Gardener 2006). Most microbes are neutralistic, therefore it is hard to screen biocontrol agents from soil and other environments.

A major group of biological control agents is plant growth promoting rhizobacteria, also known as PGPR. It is a group of bacteria that colonize at the

rhizosphere (Bhattacharyya and Jha 2012; Kumar and Sarma 2016; Siddiqui 2005). Those rhizobacteria can enhance plant growth through multiple mechanisms such as biological nitrogen fixation (mutualism), rhizosphere engineering (competition), producing antifungal compounds (antagonism), interfering with pathogen toxin production, as well as manipulating phytohormone production and inducing systemic resistance of plants (Bhattacharyya and Jha 2012; Idriss et al. 2002; Van Wees et al. 2008). Some plant extracts are also capable of stimulating the regulation of phytohormones and thus inducing of plant resistance (Su 2012). So far, few biological control agents have been tested against *Phytophthora erythroseptica*, although there has been considerable research on bio-controls of other oomycete diseases of potato (Daami-Remadi et al. 2012; Etebarian et al. 2000; Yao et al. 2016). It is possible the PGPR and plant extracts that promote potato plant growth, induce potato systemic resistance, and stimulate anti-oomycete compounds secretion can be used in pink rot management.

Crop rotation

Crop rotation is an important management method in potato pink rot control (Larkin 2008; Larkin et al. 2010; Peters 2003; Toquin et al. 2008). It replenishes soil nutrient resources, improves soil properties, reduces erosion, breaks up the life cycle of soilborne plant pathogens, and changes the abundance and diversity of the soil microbiome that favors plant health (Larkin et al. 2010; Larkin et al. 2012; Sudini et al. 2011).With the needs of reducing the use of fungicides to mitigate fungicide resistance problems, and supporting the

transition from conventional farms to organic farms, crop rotation becomes a key element in disease management (Johnson and Sideman 2006; Liebman et al. 1996; Mcgrath 2009; Vincelli 2014). In Maine, crop rotation is encouraged in potato production and disease management (Coxe and Hedrich 2007; Halloran et al. 2005; Johnson and Sideman 2006; Larkin 2014).

Crop rotation defines a system in which appropriate crops are rotated or alternated in a sequence within a period of time. It is considered as a biological disease control method depending on time, environment condition, status of pathogens and crops (El-Nazer and McCarl 1986; Narayanasamy 2013c). Therefore, the effectiveness of crop rotation varies from one field to another and from year to year. Crop rotation has direct effects on crop health; it boosts crop growth, increases crop yield and improves crop quality. Rotation crops can contribute carbon and nitrogen to succeeding crops, which may create a nutrient-rich environment for potato plants (Aubinet et al. 2009; Havlin et al. 1990; Honeycutt et al. 1996; Honeycutt 1998). Additionally, crop rotation has indirect effects on plant health; it has good performance in soilborne or plant residue-borne pathogen control. Non-host rotation crops can interrupt the lifecycles of soilborne pathogens; the absence of susceptible crops causes the failure of pathogen survival and reproduction. It is effective when the inoculum source is from the target planting site and the potential movement of the pathogen from other adjacent fields is limited (Narayanasamy 2013c). It may not be able to break up the life cycle of pathogens that produce resting spores and long-lasting survival structures, but it can change the abundance and diversity of the soil

microbiome in a way that favors plant health and suppresses the growth of target pathogens (Brewer 2003; Larkin 2008; Larkin et al. 2010; Lee Marzano et al. 2014; Sudini et al. 2011). It was also reported that crop rotation may be able to confer disease resistance to potatoes (Peters et al. 2005a).

The effects of different cropping systems on potato health and tuber yield have been extensively studied (Grandy et al. 2002; Mohr et al. 2011; Myers et al. 2008; Ryakhovskaya and Gainatulina 2009; Scholte 1987). Many crops have been found to be beneficial to potato disease control. For instance, the results in some cases showed: canola (*Brassica napus*), and rapeseed (*Brassica napus*) can reduce the severity of stem canker, black scurf, and common scab (Larkin et al. 2010); Sweet clover (*Melilotus officinalis*) and hay can reduce verticiilium wilt (Emmond 1972); Alfalfa (*Medicago sativa*) and OatsOats (*Avena sativa*) can reduce black dot of potato (Johnson and Cummings 2015). However, few studiesreveal the crop rotation effects on pink rot and the correlation between cropping sequences and soil microbial community compositions.

Some rotation crops are known to contain antimicrobial compounds that directly inhibit or reduce a wide range of bacteria and fungi including soilborne pathogens on potato (Ojaghian 2012). The *Brassica* family (e.g. canola, rapeseed) has the capability to produce glucosinolates, which will become isothiocyanates after further reactions (Ojaghian 2012). Isothiocyanates are known as biofumigants that have a suppressive effect on various soil organisms (Larkin 2007; Mazzola 2005; Ojaghian 2012). Other than glucosinolates, there might be some substances released by *Brassica* crops can also suppress fungal

pathogens (Mazzola 2005). *Allium* family (e.g. garlic, onion) is another group with the ability to release antifungal and antibacterial chemicals. Researchers found that *Allium* family can release diallyl sulfide and allicin, which are both noted for their antimicrobial activity (Benkeblia 2007). It was also reported that a PGPR (Plant growth promoting rhizobacteria) bacteria, *Rhizobium leguminosarum* bv. trifolii, is associated with clover (*Trifolium pratense*) and wheat (*Triticum spp.*.) roots (Urban 1982). It is possible that those crops can affect the soil microbial community structure and the activity of soil microbes including *P. erythroseptica,* and thus suppress the development of potato pink rot.

Fungicide Resistance in *Phytophthora*

In the 1970s, it was found that resistant mutants could be selected from the target pathogen populations that were exposed to fungicides (Brent and Hollomon 2007a; Davidse 1988, 1995; Hollomon 2015b). Fungicide resistance quickly became a concern in plant disease control. Thereupon the Fungicide Resistance Action Committee (FRAC, http://www.frac.info/) was established to prolong the effectiveness of fungicides that were liable to encounter resistance problems and to limit crop losses that might be caused by fungicide resistance (FRAC ; Hollomon 2015b). FRAC categorizes all the synthetic fungicides in groups based on the MOA of fungicides, and keeps records of resistance reports.

The presence of resistance can be a result of naturally occurring resistant individuals, which develop from a small population to be the majority in the field due to fungicide selection. Some pathogens can develop multiple-fungicide

resistances, and the fungicide resistance of these pathogens can be caused by a different fungicide (cross-resistance) (Brent and Hollomon 2007b; Gisi et al. 2000; Ishii and Holloman 2015). There are several types of mechanisms in fungicide resistance. Alteration of the biochemical target site is the most common mechanism. Synthetic fungicides have one or multiple specific target sites where they work to disrupt a particular biochemical process or function. Single-site inhibitors act on one single target site; therefore, it is easy for target pathogens to alter the target site. As a result, these fungicides lose the capability to bind to the target site and thus lose efficacy (Brent and Hollomon 1995; Buhler 2013; Deising et al. 2008). Some pathogens are insensitive to fungicides because they can increase the production of the fungicide-target protein or develop an alternative metabolic pathway to bypass the target site (Brent and Hollomon 1995). Some fungal pathogens are capable of releasing metabolites to degrade fungicides before they can reach the target sites (Brent and Hollomon 1995; Buhler 2013). Exclusion of the fungicide through ATP-ase dependent transporter proteins is another mechanism of fungicide resistance (Brent and Hollomon 1995). Sometimes in the same species, the resistant population takes up less fungicides, because they absorb fungicides more slowly than the susceptible population (Buhler 2013).

Powerful oomyceticides Phenylamides (PAs), such as mefenoxam, are single-site inhibitors (Gisi et al. 2000; Gisi and Sierotzki 2015). Thus, they have a high risk of fungicide resistance (Fungicide Resistance Action Committee 2016; Hollomon 2015a). The most effective oomycete fungicide in pink rot control,

mefenoxam (Urech et al. 1977; Wicks et al. 2000), is losing its efficacy as the mefenoxam-resistant population grows in *P. erythroseptica* (Al-Mughrabi et al. 2007; Fitzpatrick-Peabody and Lambert 2011; Torres et al. 1985; Venkataramana et al. 2010). The insensitivity of *P. erythroseptica* to mefenoxam was first reported in Maine (Lambert and Salas 1994), and then discovered in New York, Idaho, North Dakota, Minnesota etc. (Goodwin and McGrath 1995; Porter et al. 2007; Taylor et al. 2002; Venkataramana et al. 2010). Taylor et al. (2002) tested the mefenoxam sensitivity of *P. erythroseptica* isolates collected from Idaho, Maine, Minnesota, Nebraska, North Dakota, Oregon, Washington, etc. from 1997 to 2000. The results showed that the overall percentage of mefenoxam-resistant populations in isolate collections rose from 2.9% to 36.2%, and the majority of mefenoxam-resistant isolates were found in Idaho and Maine (Taylor et al. 2002). A pink rot survey conducted in 2005 revealed that over 70% of *P. erythroseptica* isolates collected from 50 local storage sites in Maine were resistant to mefenoxam, (Fitzpatrick-Peabody and Lambert 2011). Therefore, it is necessary to test new synthetic fungicides on *P. erythroseptica* and establish the sensitivity baselines to observe the pathogen response and monitor the development of fungicide resistance (Russell 2002).

Assays for fungicide sensitivity baseline

Plant pathogens may be sensitive to new chemical fungicides, but they can quickly develop resistance if the selection pressure is high. Fungicide sensitivity baselines are used to assess the risk of fungicide resistance in

pathogens. A baseline of a fungicide is a profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide. It can be used for the establishment of, and subsequent monitoring of, fungicide resistance management strategies (Russell 2002). Usually, a sample size between 20 and 50 is adequate to represent the response of the full population to a fungicide, although larger sample sizes will produce better results (Russell 2002). A typical fungicide sensitivity baseline shows the distribution of pathogen isolates on different ranges of EC50 (the dose that reduces the 50% of the full growth of an isolate, which is determined by culturing the same isolate in the absence of fungicide) or different levels of resistance (Olaya and Köller 1999; Russell 2002).

Traditionally, fungicide sensitivity is examined on agar plates amended with fungicides at various concentrations. This method is called serial dilution plating, in which the fungicide sample is diluted into a series of concentrations and incorporated into the agar medium with each plate containing a different concentration of the fungicide (Cavalieri et al. 2005). Dilution plating is a standard method used to observe the changes of pathogen growth along with the concentration of fungicides (Förster et al. 2004; Hu and Li 2014; Wexler et al. 1996). The agar dilution plating method is good for most non-fastidious organisms and provides reproducible results (Cavalieri et al. 2005). However, it has a few disadvantages including the intensive labor and time required to prepare the agar plates and their relatively short shelf life (Cavalieri et al. 2005),

as well as the inherent dilution errors that lead to inaccurate results of fungicide sensitivity (Wexler et al. 1996).

In 1990, the spiral gradient endpoint (SGE) method was introduced in antibiotics sensitivity tests (Hill and Schalkowsky 1990; Paton et al. 1990). SGE was first proposed by Spiral System Instruments Inc., and they suggested that the spiral plater (an instrument that was used for microbial enumeration) represents a potentially more efficient method of performing agar-dilution susceptibility tests (Hill and Schalkowsky 1990; Paton et al. 1990). The SGE method demonstrated an 90.7% correlation of the minimal inhibitory concentrations (MICs) with those from the traditional dilution plating method tested in parallel for 161 strains of bacteria and eight antibiotics (Hill and Schalkowsky 1990). Similar results were found by Paton et al. and other researchers (Paton et al. 1990; Pong et al. 2010; Wexler et al. 1996). Compared with the traditional method, the spiral gradient endpoint (SGE) method is more cost-efficient with regard to labor, less time and less materials.

In 2004, Förster et al. published their study on using the spiral gradient dilution method to determine the EC50 of fungicides (Förster et al. 2004). In their study, hydrophilic cellophane films were cut into strips (50 × 3 to 4 mm), sterilized, and then the cellophane strips were covered by fungal mycelia or spores. After incubation, cellophane strips were placed on the potato dextrose agar amended with a fungicide applied in a spiral gradient dilution (by a spiral plater), in a 15-cm petri dish (Förster et al. 2004). The radial growth of the controls, the location on the PDA plate where fungal growth is inhibited by 50%,

and the distance between the center of the plate and the 50% growth point were measured after 3 days of incubation. The radial distance between the zone of 50% inhibition of growth or conidial germination and the center of the plate was defined as ER (ending radius), and the corresponding fungicide concentration was defined as EC (ending concentration). TER (tail ending radius) was used to described the radial distance between the end of mycelial growth or outlier colonies in conidial streaks and the center of the plate. TEC (tail ending concentration) was the corresponding concentration of TER. The plate size, incubation time, the concentration and molecular weight of fungicide, and spiral plating mode were used in a software to calculate the EC50 of the fungicide. The fungicide sensitivity results from spiral plating assay and those from traditional plating assay were highly correlated (over 92%) (Förster et al. 2004). Fungicide sensitivity tests have been conducted on various fungi using the spiral plating method, since Förster et al. published their work in 2004 (Amiri et al. 2013; Amiri et al. 2014; Förster et al. 2007; Gachango et al. 2012b; McKay et al. 2012; Miles et al. 2014). Therefore, the reliability of spiral plating has been well-demonstrated and accepted. Considering the advantages of spiral plating over traditional dilution plating, using spiral plating method in fungicide sensitivity baseline study is recommended.

Impacts of soil microbial community on soilborne diseases

The structure, population and activity of soil microbes are closely associated with plant disease, especially soilborne diseases (Larkin and

Honeycutt 2006; Lemanceau et al. 2007; Reed and Mazzola 2015; Rosenzweig et al. 2012; Weller et al. 2002). Studies on the effects of soil microbial inhabitants on plant disease date back to the 1970s. Smith and Snyder discovered that cultivation increased the tendency of field soil to suppress Fusarium wilting of sweet potato, and they hypothesized that the increasing suppression might result from the increase of competitive microbes in soil (Smith and Snyder 1971). Later, Baker and Cook found that the suppression of wheat take-all could be transferred from one field to another (Baker and Cook 1974), and fluorescent *Pseudomonad* bacteria that were isolated from disease-suppressive field soil were antagonistic to the take-all pathogen (Cook 2003; Cook and Rovira 1976). Fluorescent pseudomonads from a fusarium-suppressive soil were also found to be effective on wheat take-all (Wong and Baker 1984). Therefore, Baker and Cook proposed the concept of biological control: using disease-suppressive microbes to control target pathogens (Baker and Cook 1974; Cook 1985). The suppressive soil studies have revealed many potential microbe-based biocontrol agents (Chet and Baker 1981; Chung and Kim 2005; Han et al. 2001; Kloepper et al. 1980; Lemanceau et al. 2007; Scher and Baker 1980; Sneh et al. 1984). Beneficial bacteria were found in many genera such as *Azospirillum, Bacillus, Pseudomonas, Rhizobium, Serratia, Stenotrophomonas,* and *Streptomyces*; beneficial fungi were found the genera *Ampelomyces, Coniothyrium, Trichoderma*, etc. (Nihorimbere et al. 2011). Most beneficial microbes act through secreting antagonistic metabolites including diffusible antibiotics and volatile organic compounds, toxins, and bio-surfactants

that inhibits the growth of plant pathogens, while some act through establishing a parasitic relationship with pathogens, which involves the production of extracellular cell wall-degrading enzymes (Nihorimbere et al. 2011). It was also discovered that some strains of fluorescent pseudomonads were able to degrade the pathogenicity factors such as toxins produced by pathogens (Haas and Défago 2005).

In 1982, Schroth and Hancock pointed out that the incorporation of one single biocontrol agent (isolated from suppressive soil) may not be successful in disease control of commercial agriculture, because of the effects (e.g. antibiosis and competition) of other soil microbes on that biocontrol agent (Schroth and Hancock 1982). Shortly after that, Mazzola started to focus on the relationship between the whole soil microbial community and plant disease (Mazzola 1999). Generally, soil microbes are categorized into three groups: plant-beneficial, plant-neutral, and plant-pathogenic microbes (Nihorimbere et al. 2011). The majority located in rhizosphere, the area around plant roots, regulated by plant root exudates (Broeckling et al. 2008; Doornbos et al. 2012). The abundance and composition of rhizosphere microbial community associated with different crops are distinctly different. Garbeva et al. investigated the soil microbial communities in response to maize (*Zea mays* L.), oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and commercial grass mix, and found the highest percentages of beneficial bacteria were in maize and grass rhizosphere, although the highest population of *Pseudomonas* detected in barley and oats rhizospheres (Garbeva

et al. 2008). Niu et al. also found significant soil microbial community shifts in different cropping systems (Niu et al. 2016).

Many researchers have contributed to the characterization of disease-associated soil microbial community in the past 20 years (Bissett et al. 2013; Mazzola 2002, 2004; Mendes et al. 2011; Reed and Mazzola 2015; Rosenzweig et al. 2012; Weller et al. 2002; Zaccardelli et al. 2013). In some studies, the relationships between potato diseases (including black scurf, stem canker, common scab, powdery scab, etc.) and soil microbial communities were bridged through cultural practices, soil amendments and crop rotation studies in potato production systems (Larkin and Halloran 2014; Larkin 2008; Larkin et al. 2010; Larkin and Honeycutt 2006; Peters et al. 2003; Trabelsi et al. 2012). Their studies demonstrated that tillage, soil amendments and some cropping systems had significant impacts on the composition of soil microbial communities.

At the soil microbiology and root diseases committee of 2016 American Phytopathology Society (APS) meeting, researchers proposed to organize special talks on the relationship of disease control and soil microbial community, and suggested that the composition or patterns of the soil microbial community should be included in soil health reports along with other common soil health parameters in the future. With more investigations on this subject, it is possible to manually modify or alter soil microbial communities to control potato soilborne diseases including pink rot through building up plant growing promoting and disease-suppressive microbial groups.

The current knowledge about the effects of soil microbial community manipulation on potato pink rot is limited. Ecological studies and molecular studies on this matter are urged to understand the relationship between the soil microbial community and pink rot disease, as well as the influence of management measures such as soil treatments, soil amendments, crop rotation, tillage and cultivation on the soil microbial community. The investigation of the disease-conducive patterns and disease-suppressive patterns of the soil microbial community will in return provide a direction in the application of biological fungicides and soil amendments, crop rotation, tillage and cultivation.

Metagenomics on microbial analysis

Soil microbial community analysis includes soil microbial composition and microbial activity, which can be achieved through culture-based methods (dilution plating), fluorescein diacetate hydrolysis, single carbon source substrate utilization analysis, fatty acid based techniques, and denaturing gradient gel electrophoresis (Bünemann et al. 2004; Gil et al. 2011; Govaerts et al. 2007; Larkin et al. 2016; Larkin 2003; Meriles et al. 2009; Qian et al. 2014). Usually, soil microbial population and composition are determined by dilution-plating of soil suspensions on various selective agar media to numerate different groups of soil microbes (Larkin et al. 2016; Larkin 2003). Fluorescein diacetate (FDA) hydrolysis is used to measure enzyme activity based on the readings on a spectrophotometer at 490 nm and a standard curve (Boehm and Hoitink 1992; Larkin 2003). Single carbon source substrate utilization analysis is based on the

capability of soil microbes to utilize a variety of sole carbon sources to measure microbial activity (Larkin 2003), and phospholipid fatty acids are utilized as chemotaxonomic markers to study active microbes in the soil community (Bünemann et al. 2004; Gil et al. 2011). There is also a method based on DNA fingerprints of soil microbes, denaturing gradient gel electrophoresis, in which dominant microbial population shows stronger bands (Smalla et al. 2001).

Traditional soil microbial community analysis techniques are costly and time-consuming, and they only provide limited information on a portion of the soil microbial community (Bünemann et al. 2004; Larkin 2003; Smalla et al. 2001). The emergence and development of next generation sequencing (NGS) enables researchers to use soil metagenomics to compare the differences between soil microbial communities and explain their influence on crop diseases and plant pathogens, which takes soil microbial community study to a new era (Caporaso et al. 2012; Coats et al. 2014; Daniel 2005; Nesme et al. 2016; Reed and Mazzola 2015; van Elsas et al. 2008). The NGS techniques allow massive DNA amplicons to be sequenced in parallel, which is more efficient in soil microbial community analysis in comparison with traditional methods (Moorthie et al. 2011; Shendure and Ji 2008). There are five platforms in NGS: 454 pyrosequencing, Illumina, SOLiD, Ion Torrent personal genome proton and PacBio RS (Hodkinson and Grice 2015; Liu et al. 2012; Quail et al. 2012). Among them, 454 pyrosequencing and Illumina are the most frequently used platforms (Luo et al. 2012).

Early soil microbial community analysis was performed on the 454 pyrosequencing platform (Rosenzweig et al. 2012; Sugiyama et al. 2010; Sul 2009). Researchers have showed more and more interests in soil microbial amplicon analysis on Illumina due to its lower cost than other platforms (Caporaso et al. 2012; Sapkota and Nicolaisen 2015; Schmidt et al. 2013). Caporaso et al. (2012) tested Illumina on microbial communities from different sources including soil, feces and human oral environment. Their sequencing results supported known biological conclusions, which demonstrated the reliability of soil microbial amplicon analysis on Illumina platform. Recently, Reed and Mazzola (2015) utilized the Illumina platform to investigate apple replant disease-associated microbial communities (Reed and Mazzola 2015).

On the Illumina Miseq platform, DNA amplicons from different samples are indexed with specific barcode sequences that are unique in each sample, which identify the samples from which the DNA amplicons originating (Illumina 2011, 2013, 2016). The pair-end sequencing allows the indexed amplicons to be sequenced from two ends while being synthesized (Illumina 2016). In the process, four fluorescently labeled nucleotides were incorporated in the synthesized DNA, and the fluorescent dyes are identified by laser excitation and imaging after incorporation (Illumina 2016). The DNA sequences generated in the process are sorted out based on the unique barcodes, and thus the sequences associated with each sample are identified.

Conclusions

Pink rot of potato caused by *Phytophthora erythroseptica* is an important disease in the United States. In the disease management of potato pink rot, synthetic fungicides have played an important role in the past. However, the development of fungicide-resistant *P. erythroseptica* poses a problem and creates a challenge in pink rot control. In the author's opinion, a potato field should be considered as a system, and integrated management should be conducted in a way that favors plant health and counters the growth of pathogens. It is highly recommended to combine methods including selecting disease-free fields, using certified seed tubers and selecting disease resistant potato cultivars, crop rotation, fertilization and irrigation management, as well as fungicide application, to suppress pink rot in potato production.

The development of integrated pink rot management requires the understanding of pink rot disease associated soil microbial community. It is necessary to investigate the influence of rotation crops on pink rot disease and the soil microbe communities to shed light on crop rotation and biological controls in potato pink rot management. The emergence and development of next generation sequencing (NGS) enables researchers to fulfill the investigation on soil microbial community associated with pink rot and different rotation crops.

Chapter 2

EFFECTS OF SYNTHETIC AND BIOLOGICAL FUNGICIDES ON PINK ROT OF POTATO

Chapter Abstract

Pink rot (*Phytophthora erythroseptica*) is a persistent disease on potatoes (*Solanum tuberosum*) in the United States. Controlling pink rot becomes a challenge as the most effective oomyceticide mefenoxam is losing its effectiveness owing to the development of resistance of *P. erythroseptica*. To evaluate some products in controlling pink rot, two greenhouse experiments and three field trials were conducted in 2014, 2015 and 2016. *Phytophthora erythroseptica* inoculum was evenly distributed in the furrows before potato seed tubers (cv. 'Russet Norkotah') were planted. Chemical products and biological control agents were applied either individually or in combinations, at planting or during the growing season. Tuber yield and pink rot disease severity were assessed at harvest. Field results showed that mefenoxam, fluopicolide, and oxathiapiprolin significantly reduced pink rot severity in the harvested tubers. Biologicals including *Bacillus subtilis* (Serenade Soil, Taegro), *Bacillus amyloliquifaciens* (Double Nickel, MBI-110), and extract of *Reynoutria sachalinensis* (Regalia) did not significantly reduce pink rot severity in the harvested tubers. The combinations of fluopicolide or oxathiapiprolin and *Bacillus sp*. significantly reduced pink rot in the harvested tubers. In greenhouse experiments, four soil drenching applications of Regalia increased tuber yield,

reduced pink rot disease severity and boosted the health of potato roots and stolon. These results suggested some alternative fungicides or strategies can be used to solve mefenoxam resistance problems in pink rot control.

Introduction

Pink rot (*Phytophthora erythroseptica*) is a ubiquitous potato disease (Porter et al. 2007; Wicks et al. 2000). It causes significant losses in the field and storage (Al-Mughrabi et al. 2007; Mills et al. 2005; Schisler et al. 2009). In 2004, the estimated loss in potato due to pink rot in New Brunswick (Canada) was more than $20 million (Al-Mughrabi et al. 2007). In the United States, pink rot is present in all potato fields (Boothroyd 1951; Carroll and Sasser 1974; Fitzpatrick-Peabody 2008; Taylor et al. 2002; Venkataramana et al. 2010; Wharton and Kirk 2009). It has been reported to be responsible for eight to nine percent loss of the total potato production in storage, and the highest disease incidence can reach 70% in the field (Benson 2008). Generally, all potato cultivars grown in North America are susceptible to pink rot (Peters 2001): highly susceptible cultivars include ‘Red LaSoda’, ‘Russet Norkotah’, ‘Goldrush’, ‘Red Gold’, ‘Warba’, ‘Norland’, and ‘Shepody’; moderately resistant cultivars include ‘Russet Burbank’, ‘Irish cobbler’, ‘Atlantic’, and ‘Pike’ (Benson 2008; Fitzpatrick-Peabody 2008).

Currently, pink rot management relies on appropriate fungicide application, water management, careful harvesting and temperature management during storage (Miller et al. 2003). There are few effective

fungicides in pink rot control (Benson 2008). The systemic chemical fungicide, mefenoxam, used to be the most effective product (Fitzpatrick-Peabody 2008; Johnson and Duniway 1997; Wicks et al. 2000). Mefenoxam is capable of translocating throughout the whole plant, and this attribute facilitates the control of *P. erythroseptica* (Cohen 1986). However, the mefenoxam-insensitive populations of *P. erythroseptica* have been a problem arising in potato production (Goodwin 1995; Lambert and Salas 1994; Lambert 1994; Taylor 2002). A survey in Idaho showed that over 70% of the *P. erythroseptica* isolates collected from 6 different sites were classified as highly resistant to mefenoxam (Porter 2007). A survey of over 50 grower storages in Maine in 2005 found that 70% of the 162 isolates were mefenoxam-resistant (Fitzpatrick 2006). The resistant population still made up 49% of the isolates even after suspending the use of mefenoxam for a few years (Fitzpatrick-Peabody 2008).

As supplements to soilborne disease management, composting and soil amendments have been widely studied and used in potato production (Larkin 2007, Ojaghian 2012). Antagonistic microbes and plant growth promoting microbes have been found during these studies and that leads to the development of biological agents to control potato pink rot (Wynn 1979, Etebarian 2000, Schisler 2009). However, sole application of biological agents may not be effective enough to provide a significant reduction of pink rot severity, therefore, the combination and integration of biological agents and pesticides have been recommended for pink rot control (Etebarian 2000, Schisler 2009).

In this study, synthetic fungicides (Orondis and Presidio) and biological fungicides (Taegro, Serenade soil, Regalia, Double nickel LC and MBI-110) were tested and compared with Ridomil Gold SL (active ingredient: mefenoxam) in *P. erythroseptica*-inoculated fields over two years. Pink rot disease severity was rated and tuber yield was assessed to demonstrate the efficacy of each fungicide treatment. Two biological fungicides MBI-110 (active ingredient: *Bacillus amyloliquefaciens* F727) and Regalia (active ingredient: extract of *Reynoutria sachalinensis*) were used in a greenhouse study to test the optimum application method and timing of those two biologicals. The objectives of this study were to 1) evaluate the efficacies of alternative synthetic fungicides and biologicals, 2) test different combinations of synthetic fungicides and biologicals, and 3) find the optimum application methods and timing of biocontrol products.

Materials & Methods

Isolates of *P. erythroseptica* used in the field trials

Seven *P. erythroseptica* isolates were collected: four were from a UMaine collection (Fitzpatrick-Peabody and Lambert 2011) and three were isolated from diseased tubers found in uninoculated fields at Aroostook Farm, Presque Isle, Maine. The pathogens were isolated from potato tubers showing pink rot symptoms. Pieces of tuber tissue were cut and surface-sterilized in 75% ethanol for 30 s, and then soaked in 0.6% sodium hypochlorite for 5 to 10 min, depending on the necrotic level of tuber tissue. After being rinsed three times in sterile distilled water, three tuber pieces were dried on sterile filter paper and placed on

a 1.5% water agar (15 g of agar powder: 1000 ml of water) plate. The pathogens isolated from the same tuber were considered as different copies of the same isolate. All the isolates were identified through polymerase chain reaction (PCR) and DNA sequencing. DNA was extracted and amplified with ITS1/ITS4 primers (White et al. 1990). The amplicons were sequenced and compared with *P. erythroseptica* sequences in NCBI database (Wheeler et al. 2007). The similarity was above 99%.

Mefenoxam (Ridomil Gold SL, Syngenta) sensitivity of seven *P. erythroseptica* isolates was tested using the dilution plating method. V8 agar plates (200 ml V8 juice, 2.5 g calcium carbonate, 1.5% agar, 800 ml distilled water) were amended with 6 concentrations of mefenoxam (V8-M plates): 0 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml. The isolates were cultured on 6 types of V8-M plates, and each had 4 replications. Five days later, the diameter of mycelial growth (the coverage of mycelia on a plate) was measured. EC50 of each isolate was calculated using R. 3.1.1. by plotting the data and fitting them to a linear regression model (RStudioTeam 2015). Isolates were divided into three groups: 1) Sensitive isolates: EC50 < 10 µg/ml. 2) Resistant isolates: EC50 > 100 µg/ml. 3) Intermediately resistant isolates: 10 µg/ml < EC50 < 100 µg/ml (Fitzpatrick-Peabody 2008; Peters et al. 2001).

Three isolates from this collection were used in the field inoculum preparation. Two mefenoxam-sensitive isolates (2/3) and one mefenoxam-resistant isolate (1/3) were used in the field inoculum preparation in 2014. In 2015, all three isolates used in the field trials were mefenoxam-sensitive.

Preparation of *P. erythroseptica* inoculum and field inoculations

Using a modified method based on Erwin's (Erwin and Ribeiro 1996), culture bags were made with 21" x10" x 5" mushroom spawn bags (Out Grow) containing 6 L of vermiculite (medium size) and 2.1 L of V8 broth (consisting of 0.6 L of V8 juice, 1.5 L of water and 7.5 g of calcium carbonate). The bags were autoclaved twice in 48 hours with a liquid 45min cycle (Amsco Lab 250 laboratory steam sterilizer). Cultures of *P. erythroseptica* were transferred to the culture bags and incubated at room temperature. During the inoculum growth stage, the moisture in the bags were monitored and adjusted to be optimum to *P. erythroseptica*. In the meantime, the mycelial growth of field inoculum was examined weekly. The concentration of oospores was monitored in late (maturing) stages. The culture bags were shaken periodically, to distribute nutrients and oxygen evenly and make them available, thus producing better mycelia extension and oospore growth. The vermiculite-based inoculum was used in field trials after four to six weeks of incubation.

Field trial experimental design and other details: 2014 field trial 1

This experiment was performed in a field (46°39'N, 68°00'W) located at Aroostook Farm, Presque Isle, Maine. A randomized complete block design was used with 4 blocks and 8 plots in each block. There were 4 rows in each plot, and the row length was 25 feet. The row-spacing was 3 feet and plant-spacing was 1 foot. There was an 8-feet gap between blocks to serve as buffer area and tractor road.

The experiment subject was potato (*Solanum tuberosum* 'Russet

Norkotah'). Before planting, 1,110 lb/a fertilizer (14-14-14 NPK, Cavendish) and 8.7 oz/a insecticide (Admire, Bayer) were used when rows were open. Inoculum of *Phytophthora erythroseptica* was evenly applied one hour before planting. The existence of oospores in field inoculum was confirmed before inoculation. At inoculation, vermiculite inoculum was mixed and applied into furrows. The two rows in the middle of each plot were inoculated with 6 L of vermiculite inoculum. The furrows were covered after planting on 4 June in 2014, which was finished soon after inoculation, to ensure the survival of pathogens.

Synthetic fungicides from Syngenta (Greensboro, NC) including Ridomil Gold SL, Ridomil Gold Bravo SC, Orondis (and biological fungicide Taegro (Novozymes & Syngenta) were tested in sole or combined applications (Table 2.1). All the fungicide treatments were compared with an untreated control in the *P. erythroseptica*-infested environment. The weather in Presque Isle was monitored during the growing season. The field was maintained with standard production practices. All plots were treated with insecticides, Admire Pro (Imidacloprid, 8.7 oz/a, Bayer), and herbicides, Roundup (Glyphosate, 25 oz/a, ScottsMiracle-Gro), as standard practice to that area. The weeds were hand-removed weekly. Foliar spray of Bravo ZN (tetrachloroisophthalonitrile, 1.5 pt/a, Syngenta) was applied with a 10-day interval during the season to prevent late blight disease. Foliar treatments were applied on 1 July, 2014. The emergence and vigor of potato were evaluated on 1 July and 22 July in 2014. Potato vines were killed on 15 September, 2014. Potato tubers in the middle two rows of the four-row plots were hand-harvested on 4 October, 2014.

Table 2.1. Treatment details in 2014 field trial 1.

Treatment Name	Active Ingredient	Application Rate	Application Method	Application Timing
Untreated Control	-	-	-	-
Ridomil Gold SL	Mefenoxam	0.42 fl oz/1000 ft	In-furrow	At planting
Ridomil Gold Bravo SC	Mefenoxam Chlorothalonil[a]	2.5 pt/a	Direct spray	Hilling
Orondis	Oxathiapiprolin	0.66 fl oz/1000 ft	In-furrow	At planting
Ridomil Gold SL	Mefenoxam	0.28 fl oz/1000 ft	In-furrow	At planting
Orondis	Oxathiapiprolin	1.32 fl oz/1000 ft	In-furrow	At planting
Ridomil Gold SL	Mefenoxam	0.55 fl oz/1000 ft	In-furrow	At planting
Orondis	Oxathiapiprolin	0.66 fl oz/1000 ft	In-furrow	At planting
Ridomil Gold SL	Mefenoxam	0.28 fl oz/1000 ft	In-furrow	At planting
Orondis	Oxathiapiprolin	9.6 fl oz/a	Direct spray	Hilling
Ridomil Gold SL	Mefenoxam	4.0 fl oz/a	Direct spray	Hilling
Taegro WP	*Bacillus sp.* FZB24[b]	0.36 oz/1000 ft	In-furrow	At planting
Orondis	Oxathiapiprolin	0.33 fl oz/1000 ft	In-furrow	At planting
Taegro WP	*Bacillus sp.* FZB24	0.36 oz/1000 ft	In-furrow	At planting
Orondis	Oxathiapiprolin	0.33 fl oz/1000 ft	In-furrow	At planting

[a] 72.0% of Chlorothalonil and 4.4% of propionic acid methyl ester.
[b] *Bacillus subtilis var. amyloliquefaciens* Strain FZB24.

Field trial experimental design and other details: 2014 field trial 2

This trial was performed in a field (46°39'N, 68°01'W) at Aroostook Farm, Presque Isle, Maine. A randomized complete block design was used, with 4 blocks and 12 plots in each block. There were 4 rows in each plot, and the row length was 25 feet. The row-spacing was 3 feet and plant-spacing was 1 foot. There was an 8-feet gap between blocks to serve as buffer area and tractor path.

The experiment subject was potato (*S. tuberosum* 'Russet Norkotah'). Before planting, 1,110 lb/a fertilizer (14-14-14 NPK, Cavendish) and 8.7 oz/a insecticide (Admire, Bayer) were used when rows were open. Inoculum of *P. erythroseptica* was evenly applied one hour before planting. The existence of oospores in field inoculum was confirmed before inoculation. At inoculation, vermiculite inoculum was mixed and applied into furrows. The two rows in the middle of each plot were inoculated with 6 L of vermiculite inoculum. The furrows were covered after planting, which was finished soon after inoculation, to ensure the survival of pathogens. Above-mentioned activities were done on 4 June, 2014.

Three biological agents, Regalia (Marrone, Bio Innovations Inc., Davis, CA), Serenade Soil (Bayer, Research Triangle Park, NC) and MBI110 (Marrone) were tested in the field. MBI110 is derived from a strain of *Bacillus amyloliquefaciens*, and it will be a commercial product soon. In this field trial, two fungicides Ridomil SC (Syngenta, Greensboro, NC) and Presidio (Valent, Walnut Creek, CA) were used as standards to evaluate the efficacy of biological products and the combinations of synthetic and biological fungicides (Table 2.2.).

Table 2.2. Treatment details in 2014 field trial 2.

Treatment Name	Active Ingredient	Application Rate	Application Method	Application Timing
Untreated Control	-	-	-	-
MBI-110	*Bacillus sp.* F727[a]	6 qt/a	In-furrow	At planting
Regalia	Plant extract[b]	4 qt/a	In-furrow	At planting
Serenade Soil	*Bacillus sp.* QST 713[c]	6 qt/a	In-furrow	At planting
Presidio	Fluopicolide	13.7 fl oz/a	In-furrow	At planting
Ridomil Gold SC	Mefenoxam	0.42 fl oz/a	In-furrow	At planting
Ridomil Gold SC Presidio	Mefenoxam Fluopicolide	0.42 fl oz/a 4 fl oz/a	In-furrow Foliar	At planting Nickel tuber
Ridomil Gold SC MBI-110	Mefenoxam *Bacillus sp.* F727	0.42 fl oz/a 6 qt/a	In-furrow Foliar	At planting Nickel tuber
Ridomil Gold SC Serenade Soil	Mefenoxam *Bacillus sp.* QST 713	0.42 fl oz/a 6 qt/a	In-furrow Foliar	At planting Nickel tuber
Ridomil Gold SC Regalia	Mefenoxam Plant extract	0.42 fl oz/a 4 qt/a	In-furrow Foliar	At planting Nickel tuber
Presidio MBI-110	Fluopicolide *Bacillus sp.* F727[a]	13.7 fl oz/a 6 qt/a	In-furrow Foliar	At planting Nickel tuber

[a] *Bacillus amyloliquefaciens* strain F727.
[b] Extract of *Reynoutria sachalinensis.*
[c] *Bacillus subtilis* strain QST 713.

The weather in Presque Isle was monitored during the growing season. The field was maintained with standard production practices. All plots were treated with insecticides Admire Pro (8.7 oz/a), and herbicides Roundup (25 oz/a), as standard practice to that area. The weeds were hand-removed weekly. Bravo ZN (1.5 pt/a, Syngenta) was applied weekly on foliar for late blight control starting from 24 Jul to 11 Sep. Foliar application of treatments, such as Presidio, MBI-110, Serenade Soil and Regalia were applied weekly starting from 24 Jul until 11 Sep, 2014. The emergence and vigor of potato plants in this trial were not recorded. Potato vines were killed by applying Reglone (1.5 lb/a) on 15 and 20 September, 2014. Potato tubers in the middle two rows of the four-row plots were dug with a 2-row harvester on 4 October, 2014. Pink rot severity and yield data were collected on 13 October, 2014.

Field trial experimental design and other details: 2015 field trial

This experiment was carried out in a field (46°65'N, 68°01'W) at Aroostook Farm, Presque Isle, Maine. A randomized complete block design was used, with 4 blocks and 11 plots in each block. There were 4 rows in each plot, and the row length was 35 feet. The row-spacing was 3 feet and plant-spacing was 1 foot. There was an 8-feet gap between blocks to serve as buffer area and tractor path.

The experiment subject was potato (*S. tuberosum* 'Russet Norkotah'). Before planting, 1,110 lb/a fertilizer (14-14-14 NPK, Cavendish) and 8.7 oz/a insecticide (Admire, Bayer) were used when rows were open. Inoculum of *Phytophthora erythroseptica* was evenly applied one hour before planting. The existence of oospores in field inoculum was confirmed before inoculation. At

inoculation, vermiculite inoculum was mixed and applied into furrows. The two rows in the middle of each plot were inoculated with 6 L of vermiculite inoculum. The furrows were covered after planting, which was finished soon after inoculation, to ensure the survival of pathogens. Above-mentioned activities were done on 25 May, 2015.

Five chemical fungicides and two biological fungicides were tested in this trial. Ridomil Gold SL was used as standard control. The combination of presidio (at reduced rate) and MBI-110 was tested to confirm the results observed in 2014 trial 2. Two application arrangements were used for MBI-110 to find the most appropriate application amount (Table 2.3.).

The weather in Presque Isle was monitored during the growing season. The field was maintained with standard production practices. All plots were treated with insecticides, Admire Pro (8.7 oz/a), and herbicides, Roundup (25 oz/a), as standard practice to that area. The weeds were hand-removed weekly. The treatments were applied at three timings: in-furrow at planting (May 25), dime tuber size (July 10), and two weeks after the dime tuber (July 22). All the plants were treated with Bravo ZN (1.5 pt/a) during the season to control late blight. Plant emergence and vigor were evaluated on 26 June and 16 July. Potato vines were killed by Reglone application (1.5 lb/a) on 7 September, 2015. Potato tubers in the middle row of each treatment were dug by a harvester and hand-picked on 24 September, 2015. Pink rot disease severity was rated and tuber yield was weighed on 11 October, 2015.

Table 2.3. Treatment details in 2015 field trial.

Treatment Name	Active Ingredient	Application Rate	Application Method	Application Timing
Untreated Control	-	-	-	-
Ridomil Gols SL Phostrol	Mefenoxam phosphorous acids	0.4 fl oz/1000 ft 10 pt/a	In-furrow Spray	At planting Dime tuber[a]
Presidio Phostrol	Fluopicolide phosphorous acids	4.0 fl oz/a 10 pt/a	In-furrow Spray	At planting Dime tuber
Emesto Silver Reason	Penflufen & Prothioconazole Fenamidone	0.31 oz/cwt[b] 0.15 oz.cwt	Seed treatment	Pre-planting
Double Nickel LC	*Bacillus sp.* D747[c]	1 qt/a	In-furrow	At planting
Presidio MBI-110	Fluopicolide *Bacillus sp.* F727[d]	2.0 fl oz/a 6 qt/a	In-furrow Soil drenching	At planting Four times from tuber initiation
MBI-110	*Bacillus sp.* F727	6 qt/a	Soil drenching	Once at tuber initiation
MBI-110	*Bacillus sp.* F727	6 qt/a	Soil drenching	Four times from tuber initiation

[a] Applicated twice: applied at dime tuber stage and a week after the first application.
[b] cwt: 100 lb of seed tubers.
[c] *Bacillus amyloliquefaciens* strain D747.
[d] *Bacillus amyloliquefaciens* strain F727.

Plant emergence and vigor evaluation

During the growth season, plant emergence and vigor were estimated and recorded periodically. Emerged plants of the middle rows were counted. Plant emergence was calculated based on the percentage of emerged seeds out of total number of seed tubers in that row (Eq.2.1.). Relative plant vigor was

estimated, based on the plot with the best vigor (100%) in each block, expressed by percentages.

Eq.2.1. Plant emergence calculation

$$\text{Plant emergence} = \frac{\text{the number of emerged plants in a row}}{\text{total number of seed tubers in a row}} \times 100\%$$

Preparation of *P. erythroseptica* inoculum and greenhouse inoculations

Three mefenoxam-sensitive *P. erythroseptica* isolates were used in greenhouse trials. In 2015, vermiculite inoculum was prepared as described earlier in this thesis (See "*Preparation of P. erythroseptica inoculum and field inoculations*"), and used in MBI-110 and Regalia experiments. The regalia trial was repeated in 2016, and zoospore suspension was used as the primary inoculum (Al-Mughrabi et al. 2007). Zoospores of *P. erythroseptica* were produced following the method published by Al-Mugharbi et al. (Al-Mughrabi et al. 2007), modified by Jiang (unpublished).

To prepare zoospores, *P. erythroseptica* was cultured on 10% V8 (see Appendix A) agar (25 °C, dark) for 6 days. Four mycelial plugs were punched by a 5-mm cork borer and transferred to a petri plate (10 mm diameter) filled with 10 ml of lima bean broth (LBB, see Appendix A). After incubation at 22 °C under natural light for 3 days, LBB was discarded. The original (10% V8) agar plugs were removed, and the mycelial mats were rinsed with sterilized distilled water three times. The Petri plates (with mycelial mats only) was filled with 10 ml of 10% soil extraction (see Appendix A), and incubated at 18 °C under continuous fluorescent light for 4 days (Al-Mughrabi et al. 2007). Four days later, the Petri

plate and mycelial mats were rinsed again with sterile water (4 °C, cold water), and 10% soil extraction was replaced by 7 ml of sterile water (4 °C). The plate was chilled at 4 °C for an hour and then left at 22 °C for 30 min. Then the zoospore concentration was estimated with a hemocytometer.

Greenhouse trial experimental design

To explore further information about the optimum application method and timing, greenhouse experiments on MBI-110 and Regalia were performed in Room 2 of Roger Clapp greenhouse at University of Maine (Orono, ME). The greenhouse trials were first conducted in 2015, and Regalia trial was repeated in 2016 owing to missing data in 2015 Regalia trial. A randomized complete block design was used in all the greenhouse experiments to rule out the bench position effects.

Potato seed tubers (c.v. Russet Norkotah) were cut and left curing overnight. Each treatment had 5 replications, and was randomly assigned to a pot. All the plants or pots were independent. The spacing between two plots was 1 foot. One pot contained 1.5 gallon of soil mixture (field soil: potting mix: vermiculite= 1:1:1).

In 2015, vermiculite inoculum was used. Each experimental plant was inoculated with 120 ml of vermiculite inoculum at planting. In 2016, experimental plants were inoculated with zoospore inoculum by injecting it into plant root zone at the third week after planting. The inoculation method was adapted from Al-Mughrabi et al.'s method (Al-Mughrabi et al. 2007). Pipette tips were used to punch a hole (the same depth as planting) on each side of the plant next to the

plant root zone. Five milliliter of zoospore inoculum ($1x10^3$ spores/ml) was injected into each hole using a pipette and the holes were covered by the soil. The pot soil was wetted before and 24 h after inoculation to ensure that zoospores could swim to belowground plant tissues to cause infection (Al-Mughrabi et al. 2007).

All the treatments were applied directly in planting holes or through soil drenching. In 2015 Regalia experiments, the first application started at planting, but in 2016 Regalia experiments, the first application started at 24 h after planting. Each treatment was added into 100 ml of water and applied to each individual plant. Presidio was used as standard control in all the greenhouse experiments. MBI-110 (Table 2.4.) or Regalia was applied at different timing in different ways (Table 2.5.).

Table 2.4. Treatment details in MBI-110 greenhouse trial.

Treatment Name	Active Ingredient	Application Rate[a] (ml/pot)	Application Method	Application Timing
Uninoculated control	-	-	-	-
Inoculated control	-	-	-	-
MBI-110	*Bacillus sp.* F727[b]	0.0578	In-hole	At planting[c]
MBI-110	*Bacillus sp.* F727	0.0578	Soil drenching	Tuber initiation[d]
Presidio	Fluopicolide	0.0041	In-hole	At planting
Presidio	Fluopicolide	0.0041	Soil drenching	Tuber initiation
Presidio MBI-110	Fluopicolide *Bacillus sp.* F727	0.0041 0.0578	In-hole Soil drenching	At planting Tuber initiation
Presidio (half rate) MBI-110	Fluopicolide *Bacillus sp.* F727	0.0021 0.0578	In-hole Soil drenching	At planting Tuber initiation
Ridomil Gold 480 SL	Mefenoxam	0.0092	In-hole	At planting

[a] Converted based on the surface area of each pot.
[b] *Bacillus amyloliquefaciens* strain F727.
[c] One application at planting.
[d] Four applications starting from tuber initiation with a 10-day interval.

Table 2.5. Treatment details in Regalia trials.

Treatment Name	Application Rate[a] (ml/pot)	Application Method	Application Timing
Uninoculated control	-	-	-
Inoculated control	-	-	-
Presidio[b]	0.0041	In-hole /drenching	Once, at/24 h after planting
Regalia[c]	0.0385	In-hole /drenching	Once, at/24 h after planting
Regalia	0.0385	Soil drenching	Once, 10 d after emergence
Regalia	0.0385	Soil drenching	Once, 20 d after emergence
Regalia	0.0385	Soil drenching	Once, 40 d after emergence
Regalia	0.0385	Soil drenching	Four applications: At/24 h after planting, 10 d, 20 and 40 d after emergence

[a] Converted based on the surface area of each pot
[b] Active ingredient: fluopicolide
[c] Extract of *Reynoutria sachalinensis*

Assessment of pink rot disease and potato tuber yield in field trials

At harvest, tubers from the middle 2 rows were dug out and left in separate containers that had been tagged with treatment labels. The tubers were sized (US1: 2 inch minimum, US2: 1.5 inch minimum) and weighed for commercial yield (US1) and marketable yields (sum of US1 and US2) (Keough 2016). In 2014, only one of the middle two rows of each plot was used for data

collection. However, in 2015, all the tubers were weighed to collect yield data, only one row was used for disease evaluation. Tuber yield and disease severity data were collected traditionally (Larkin 2007; Larkin et al. 2010; Lobato 2008). Pink rot severity was rated by reading the approximate coverage of the visible symptom (pink stain) on the cut surface of tubers, based on a scale of 0 = no symptoms, 1 = < 2.5% of surface with symptoms, 2 = 2.5 – 10% surface with symptoms, 3 => 10 – 25% surface with symptoms, 4 => 25–50% surface with symptoms and 5 => 50% surface with symptoms of susceptibility. Disease index was calculated using the following formula (Eq.2.2):

Eq.2.2.

$$\text{Disease Index} = \frac{\text{Sum of all disease ratings}}{\text{Total number of tubers} \times \text{maximum rating value}} \times 100\%$$

Assessment of pink rot disease and potato tuber yield in greenhouse trials

In greenhouse studies, the total weight of tubers per pot was considered as tuber yield. All the harvested tubers were examined for pink rot severity at harvest time. The disease evaluation method was the same as field disease assessment.

Data analysis of fungicide trials

The yield and disease severity data were analyzed using R. 3.1.1. and JMP 9.0. (SAS Institute, Cary, North Carolina). The means values were

compared using nonparametric test with Wilcoxon each pair comparison, or ANOVA with fisher's LSD, at $\alpha = 0.05$.

Results

Mefenoxam sensitivity of *Phytophthora erythroseptica* isolates

In the dilution plating assay, seven isolates were cultured on V8-M plates with 6 concentrations of mefenoxam (0 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml), with 4 replications. EC50 calculation showed that one isolate (13A02) was resistant to mefenoxam, and the rest were sensitive to mefenoxam. Among them, 13A09 was extremely susceptible to mefenoxam.

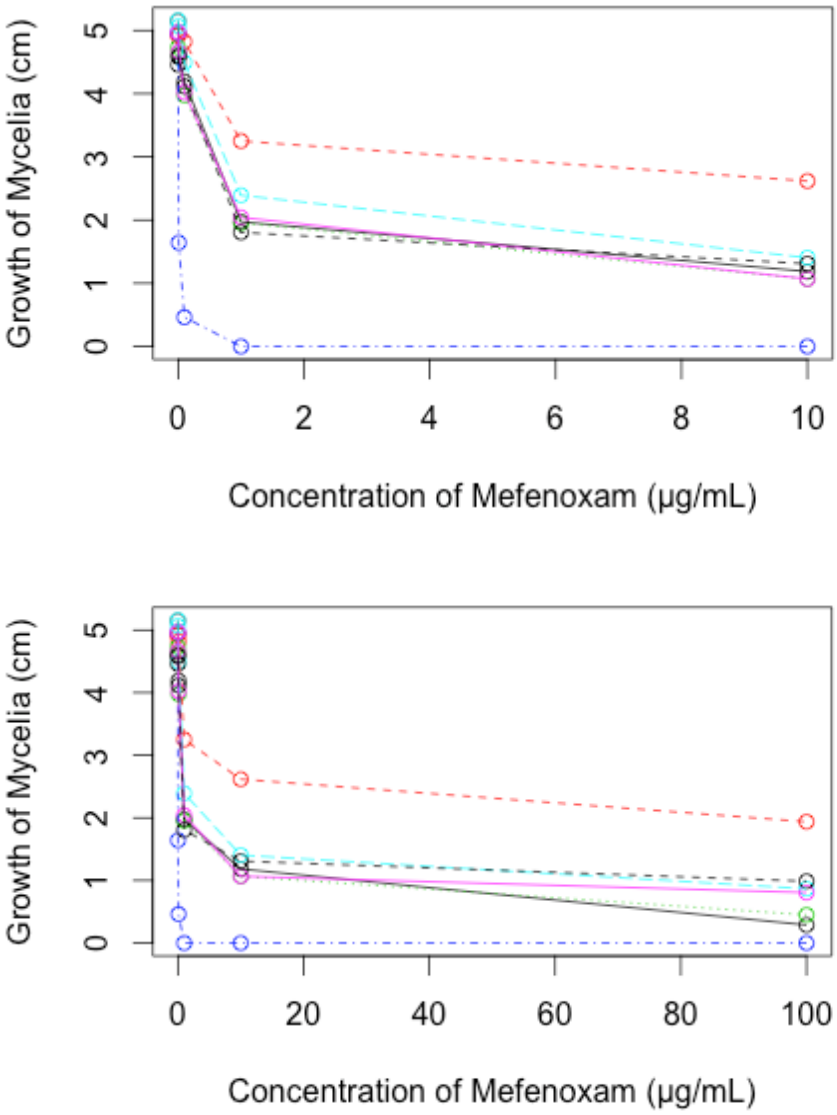

Figure 2.1. Sensitivity of *Phytophthora erythroseptica* isolates to mefenoxam.

Red broken line indicates isolate 13A02. Light blue broken line, 16A12. Black broken line, 16A14. Pink solid line, 16A13. Green broken line,13A06. Grey solid line,13A01. Blue broken line, 13A09.

Plant emergence and vigor data in 2014 field trial 1

In 2014 field trial 1, plant emergence and vigor was evaluated twice. The initial data were collected on 1-July, about 4 weeks after planting. The results (Table 2.6) showed that all the treatments except Taegro increased the emergence and enhanced plant vigor significantly in comparison with the untreated control ($\alpha = 0.05$). Taegro did not have a significant effect on emergence, but it had a negative effect on plant vigor ($\alpha = 0.05$). Plant emergence and vigor was evaluated again on 22-July. It was noticed that all the fungicide treatments except “Taegro” had positive effects on plant emergence and vigor ($\alpha = 0.05$). Among them, In-furrow application plus direct spraying at hilling of Orondis and Ridomil Gold SL provided the best results. Compared with the untreated control, “Taegro” decreased plant emergence, although it did not have a significant effect on plant vigor ($\alpha = 0.05$). Overall, all the treatments with synthetic fungicides (Ridomil Gold SL, Ridomil Bravo SC or Orondis) including “Orondis +Taegro” (the combination of synthetic and biological fungicides) improved plant emergence and vigor. “Taegro” seemed to have a negative effect on plant emergence and vigor.

Table 2.6. Plant emergence (%) and vigor (%) in 2014 field trial 1.

Treatment	July 1		July 22	
	Emergence	Vigor	Emergence	Vigor
Untreated	56.9 b[a]	75.8 c	77.5 c	73.8 d
Ridomil Gold 480 SL 0.42 fl oz/1000 ft, furrow + Ridomil Gold Bravo SC, 2.50 pt/a, hilling	77.5 a	87.0 b	86.9 b	89.8 bc
Orondis 0.66 fl oz/1000 ft, furrow + Ridomil Gold 480 SL 0.28, furrow	84.4 a	88.5 ab	91.9 ab	87.0 c
Orondis 1.32, fl oz/1000 ft furrow + Ridomil Gold 480 SL 0.55, furrow	80.0 a	85.3 b	91.3 ab	92.0 abc
Orondis 0.66 fl oz/1000 ft furrow + Ridomil Gold 480 SL 0.26 fl oz/1000 ft, furrow + Orondis 9.6 fl oz/1000 ft hilling + Ridomil Gold 480 SL 4.00 fl oz/1000 ft, hilling	84.4 a	88.3 ab	95.6 a	98.8 a
Taegro 13 WP 0.36 oz/1000 ft, furrow + Orondis 0.33 fl oz/1000 ft, furrow	80.0 a	88.3 ab	93.8 ab	94.5 abc
Taegro 13 WP 0.36 oz/1000 ft, furrow	55.6 b	67.5 d	68.8 d	65.0 d
Orondis 0.33 fl oz/1000 ft, furrow	87.5 a	93.8 a	95.0 ab	96.5 ab

[a] Mean values connected by the same letters are not significantly different (α = 0.05).

Plant emergence and vigor data in 2015 field trial

In 2015 field trial (Table 2.7), none of the fungicide treatments had a significant effect on plant vigor in comparison with untreated control (α = 0.05). However, the seed treatment (Emesto Silver plus Reason) significantly increased plant emergence (α = 0.05). Soil treatments (Ridomil gold SL, presidio and Double Nickel LC) did not affect plant emergence and vigor.

Table 2.7. Plant emergence (%) and vigor (%) in 2015 field trial.

Treatments	June 26		July 16
	Emergence	Vigor	Emergence
Non-treated	77.14 bc[a]	82.50 ab	80.00 b
Ridomil Gold SL 0.42 fl oz/1000 ft, furrow + Phostrol 10 pt/a, spray	72.14 c	81.25 ab	77.14 b
Presidio 4 fl oz/a, furrow + Phostrol 10 pt/a, spray	77.14 bc	82.50 ab	85.00 ab
Emesto Silver 0.31 oz/cwt, seed + Reason 0.15 oz/cwt, seed	93.57 a	97.50 a	94.29 a
Double Nickel LC 1qt/a, furrow	80.71 abc	81.25 b	82.14 ab
Presidio 2 fl oz/a, furrow + MBI-110 6 qt/a, drenching (4 times since tuber initiation)	85.71 ab	90.00 ab	79.29 b
MBI-110 6 qt/a, drenching (once at tuber initiation)	76.43 bc	82.50 ab	77.14 b
MBI-110 6 qt/a, drenching (4 times since tuber initiation)	76.43 bc	85.00 ab	80. 72 b

[a] Mean values connected by the same letters are not significantly different (α = 0.05).

Assessment of pink rot disease and tuber yield in 2014 field trial 1

In 2014 field trial 1 (Table 2.8), all the treatments involved with synthetic fungicide Orondis significantly increased potato tuber yield ($\alpha = 0.05$). Ridomil gold SL plus Ridomil gold bravo did not have a treatment effect on yield. The sole application of biological product Taegro did not have a significant effect on tuber yield. However, the combination of Orondis and Taegro significantly increased the yield and suppressed pink rot. All the treatments except “Ridomil gold SL plus Ridomil gold bravo”, Taegro and Orondis reduced potato pink rot significantly ($\alpha = 0.05$). When compared with the lower rate (Orondis 0.66 fl oz/1000 ft + Ridomil Gold 480 SL 0.28 fl oz/1000 ft) of Orondis-Ridomil treatment, the higher rate (Orondis 1.32 fl oz/1000 ft + Ridomil Gold 480 SL 0.55 fl oz/1000 ft) of Orondis-Ridomil combination caused a significant yield reduction in the harvested tubers ($\alpha = 0.05$).

Table 2.8. Tuber yield and pink rot disease index in 2014 field trial 1.

Treatment	Commercial yield (lb)	Marketable yield (lb)	Pink rot Disease Index
Untreated	20.44 d[a]	22.21 c	16.8 ab
Ridomil Gold 480 SL 0.42 fl oz/1000 ft, In-furrow + Ridomil Gold Bravo SC 2.50 pt/a, hilling	22.34 d	25.10 c	27.9 a
Orondis 0.66 fl oz/1000 ft, in-furrow + Ridomil Gold 480 SL 0.28 fl oz/1000 ft, in-furrow	37.06 a	40.91 a	0.4 c
Orondis 1.32 fl oz/1000 ft, in-furrow + Ridomil Gold 480 SL 0.55 fl oz/1000 ft, in-furrow	30.02 c	35.03 b	2.2 c
Orondis 0.66 fl oz/1000 ft, in-furrow + Ridomil Gold 480 SL 0.28 fl oz/1000 ft, in-furrow Orondis 9.6 fl oz/a hilling + Ridomil Gold 480 SL 4.00 fl oz/a, hilling	35.65 ab	40.31 a	1.3 c
Taegro 13 WP 0.36 oz/1000 ft, in-furrow + Orondis 0.33 fl oz/1000 ft, in-furrow	32.42 bc	36.78 ab	1.8 c
Taegro 13 WP 0.36 oz/1000 ft, in-furrow	18.68 d	20.28 c	29.5 a
Orondis 0.33 fl oz/1000 ft, in-furrow	31.22 bc	36.20 ab	9.9 bc

[a] Mean values connected by the same letters are not significantly different (α = 0.05).

Assessment of pink rot disease and tuber yield in 2014 field trial 2

Three biological agents (Regalia, Serenade Soil and MBI-110) and a synthetic fungicide Presidio were tested in comparison with untreated control and Ridomil gold SC in this trial. The results showed that the sole application of biological agents did not have a significant effect on tuber and pink rot disease ($\alpha = 0.05$). Ridomil gold SC and the combinations of Ridomil and biologicals did not reduce pink rot or increase tuber yield. Presidio, and the combination of Presidio and MBI-110 significantly suppressed pink rot disease ($\alpha = 0.05$).

Table 2.9. Tuber yield and pink rot disease index in 2014 field trial 2.

Treatment	Commercial Yield (lb)	Marketable yield (lb)	Pink rot Disease index
Untreated	23.61 a	26.98 a	8.47 a[a]
MBI-110, 6 qt/a, furrow	27.72 a	28.42 a	5.39 abc
Regalia 4 qt/a, furrow	28.94 a	31.63 a	4.60 abc
Serenade Soil 6 qt/a, furrow	23.64 a	26.73 a	8.54 a
Presidio 13.7 oz/a, furrow	35.52 a	41.10 a	1.65 bc
Ridomil Gold SC 0.42 oz/a, furrow	28.36 a	32.48 a	4.84 abc
Ridomil Gold SC, furrow + Presidio 4 oz/a, foliar	31.16 a	37.00 a	3.80 abc
Ridomil Gold 0.42 oz/a, furrow+ MBI-110 6 qt/a, foliar	32.75 a	37.82 a	3.82 abc
Ridomil Gold 0.42 oz/a furrow+ Serenade Soil 6 qt/a, foliar	29.69 a	32.66 a	6.34 ab
Ridomil Gold 0.42 oz/a furrow+ Regalia 4 qt/a, foliar	32.32 a	37.05 a	4.42 abc
Presidio 13.7 oz/a, furrow+ MBI-110 6 qt/a, foliar	33.34 a	40.88 a	0.65 c

[a] Means not connected by the same letter are significantly different (α = 0.05).

Assessment of pink rot disease and tuber yield in 2015 field trial

In 2015 field trial (Table 2.10), two biological fungicides Double Nickel LC and MBI-110 were tested. Neither of them had a significant effect on tuber yield and pink rot disease ($\alpha = 0.05$). The seed treatment “Emesto Silver plus Reason” did not show a treatment effect on yield and disease reduction. Synthetic fungicide treatments (Ridomil Gold SL and Presidio) followed by Phostrol increased tuber yield and reduced pink rot disease ($\alpha = 0.05$). The combination of Presidio (at half rate) and MBI-110 suppressed pink rot and increased marketable yield, although it did not have a significant effect on commercial tuber yield ($\alpha = 0.05$).

Table 2.10. Tuber yield and pink rot disease index in 2015 field trial

Treatment	Commercial Yield (lb)	Marketable yield (lb)	Pink rot disease index
Non-treated	49.10 cd	66.88 c	25.64 a[a]
Ridomil Gold SL 0.42 fl oz/1000 ft, furrow + Phostrol 10 pt/a, spray	65.91 ab	87.74 ab	1.58 cd
Presidio 4 fl oz/a, furrow + Phostrol 10 pt/a, spray	74.68 a	96.22 a	1.23 d
Emesto Silver 0.31 oz/cwt, seed + Reason 0.15 oz/cwt, seed	59.87 abcd	80.57 abc	12.38 ab
Double Nickel LC 1qt/a, furrow	50.17 bcd	74.17 bc	16.59 ab
Presidio 2 fl oz/a, furrow + MBI-110 6 qt/a, drenching (4 times since tuber initiation)	65.00 abc	88.43 ab	10.11 bcd
MBI-110 6 qt/a, drenching (once at tuber initiation)	45.38 d	65.36 c	18.18 ab
MBI-110 6 qt/a, drenching (4 times since tuber initiation)	60.15 abcd	83.11 abc	15.06 ab

[a] Mean values connected by the same letters are not significantly different (α = 0.05).

Effects of MBI-110 on potato pink rot and tuber yield in greenhouse trial

In 2015, different application methods and timings of MBI-110 and Presidio were tested in a greenhouse experiment (Table 2.11). Ridomil Gold SL was used as standard fungicide control. There were dead plants and plants without daughter tubers in most treatments. The meanings of statistical analyses on pink rot disease index could not be determined, therefore ANOVA on pink rot disease index was not performed on the data. However, it was likely that Ridomil Gold SL and the combination of Presidio and MBI-110 increased tuber yield (α = 0.05).

Table 2.11. Tuber yield and pink rot evaluation in MBI-110 greenhouse trial

Treatment name	Tuber yield (g)	Disease index of pink rot
Inoculated control	0.00 c[a]	0
Uninoculated control	33.48 a	0
Ridomil Gold 480 SL, at planting	31.11 a	0
Presidio at planting	15.97 abc	0
Presidio, tuber initiation	19.90 abc	0
MBI-110, at planting	9.77 abc	75
MBI-110, tuber initiation	0.00 c	0
Presidio (half rate), at planting + MBI-110, tuber initiation	16.33 abc	0
Presidio at planting + MBI-110, tuber initiation	25.50 ab	0

[a] Mean values connected by the same letters are not significantly different (α = 0.05).

Effects of Regalia on potato pink rot and tuber yield in greenhouse trials

In 2015, different application methods and timings of Regalia were tested in a greenhouse experiment (Table 2.12). Presidio was used as standard fungicide control. There were dead plants and plants without daughter tubers in most treatments. The meanings of statistical analyses on pink rot disease index could not be determined, therefore ANOVA on pink rot disease index was not performed on the data. Diseased tubers were not found. However, the results indicated a trend that early applications and four applications of Regalia could increase tuber yield.

This experiment was performed again in 2016 (Table 2.13). A different inoculation method was used and data from all the pots were collected. The results confirmed the trend that was observed in 2015, early applications of Regalia could increase tuber yield, although there was no significance difference ($\alpha = 0.05$). Four-time application of Regalia (24 h after planting, 10 days, 20 days and 40 days after emergence) increased potato tuber yield and reduced pink rot ($\alpha = 0.05$). It was also noticed that four application of Regalia kept mother tubers fresh (Fig.2.2 -2.3.), and this treatment enlarged plant roots and stolon (Fig.2.4.).

Table 2.12. Tuber yield and pink rot disease index in 2015 Regalia greenhouse trial

Treatment name	Tuber yield (g/pot)
Uninoculated control	21.06 abc[a]
Inoculated control	0.00 bc
Presidio，once at planting	6.00 abc
Regalia, once at planting	27.33 ab
Regalia, once, 10 d after emergence	13.17 abc
Regalia, once, 20 d after emergence	6.00 abc
Regalia, once, 40 d after emergence	0.00 c
Regalia, four applications: At planting, 10 d, 20 and 40 d after emergence	32.54 a

[a] Mean values connected by the same letters are not significantly different (α = 0.05).

Table 2.13. Tuber yield and pink rot disease index in 2016 Regalia greenhouse trial

Treatment name	Tuber yield (g/pot)	Pink rot disease index
Uninoculated control	64.30 bc[a]	0 a
Inoculated control	30.18 c	34.00 b
Presidio，once, 24 h after planting	45.82 bc	13.33 ab
Regalia, once, 24 h after planting	80.39 bc	10.00 ab
Regalia, once, 10 d after emergence	78.77 bc	8.00 ab
Regalia, once, 20 d after emergence	47.28 bc	8.00 ab
Regalia, once, 40 d after emergence	56.25 c	20.00 ab
Regalia, four applications: 24 h after planting, 10 d, 20 and 40 d after emergence	123.22 a	0 a

[a] Mean values connected by the same letters are not significantly different (α = 0.05).

Figure 2.2. Mother tubers dug out at harvest.

A: the mother tuber from the Presidio treatment decayed. B: the mother tuber from the Regalia (four applications) treatment stayed fresh. C: The cut surface of the mother tuber from the four-application Regalia treatment did not change its color or shape after planting.

Figure 2.3. Harvested tubers from the four-application Regalia treatment “24 h after planting, 10 d, 20 d and 40 d after emergence”.

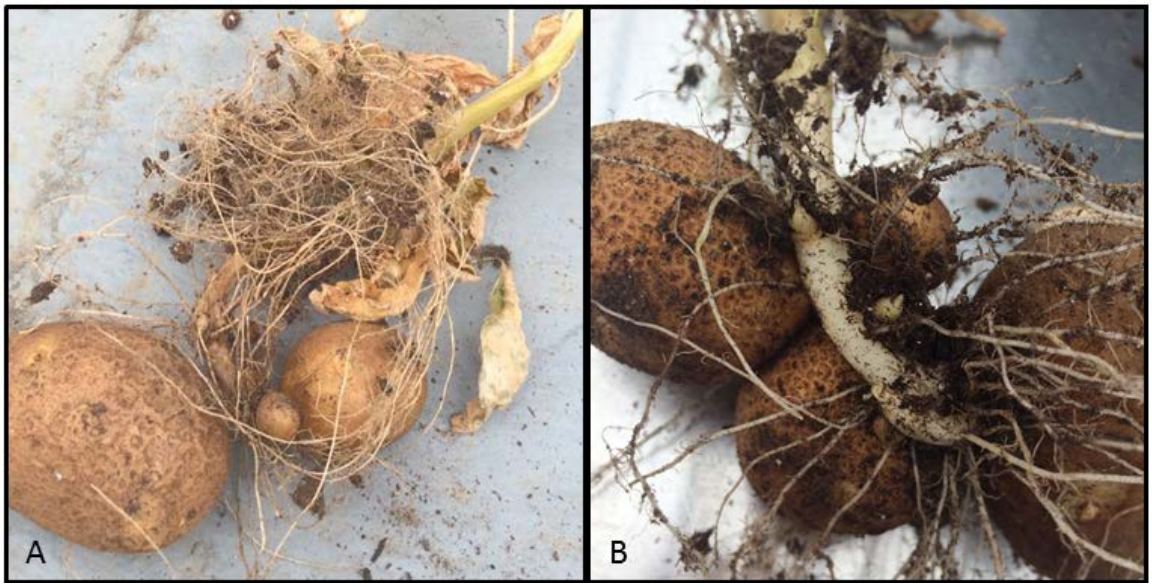

Figure 2.4. Four-application Regalia treatment kept underground plant system fresh (B), compared with non-treated control (A).

Discussion

Alternatives to mefenoxam in pink rot control

Synthetic fungicide fluopicolide reduced pink rot and increased tuber yield in all the field trials, although it did not show any treatment effects in greenhouse experiments. The target site of fluopicolide is a spectrin-like protein, which is different from the target site of mefenoxam (Toquin et al. 2008). The field results in this study also showed that there was no cross-resistance between fluopicolide and mefenoxam. Therefore, fluopicolide can be used in the fields where the mefenoxam-resistant population of *P. erythroseptica* is dominant.

The sole application of mefenoxam did not suppress pink rot in 2014 field trials because of the mefenoxam-resistant population in the inoculum. Combinations of mefenoxam and oxathiapiprolin significantly reduced pink rot severity and increased potato tuber yield in the presence of mefenoxam-resistant *P. erythroseptica*, even when the application rates were reduced. The possible reasons for the failure of Orondis treatment (sole application, ¼ of full rate) could be that the application rate was not high enough to control pink rot. The combinations of Orondis at a higher rate and Ridomil Gold suppressed pink rot and increased tuber yield.

Efficacy of biological fungicides

Five biological fungicides Taegro, Serenade Soil, Regalia, Double Nickel LC and MBI-110, were tested in this study. Taegro, Serenade Soil, Double Nickel LC are derived from different strains of beneficial bacteria *Bacillus subtilis* and *Bacillus amyloliquefaciens*. The active ingredient of Regalia derived from

Japanese knotweed *Reynoutria sachalinensis*. The field results showed that sole applications of these biological fungicides did not have a significant effect on pink rot or potato tuber yield.

There were many factors influencing the performance of biological agents in the field. Most biological agents were derived from bacteria, which require the environmental conditions to be conducive for them to establish and colonize in soil (Cabrefiga et al. 2014; De Curtis et al. 2012; Usta 2013). Available water and oxygen in soil are important for the survival of Bacteria, and that is associated with soil porosity and soil types (Parnell et al. 2016). Soil pH is another major factor that influences the performance of bacterial biological agents. The ideal soil pH for potato growth is between 5.2-6.4 (FAO 2008), and *Bacillus spp*. are known to have the best antibiotic or enzyme activity at a higher pH (7-9) (Deb et al. 2013; Jamil et al. 2007; Sudhakar et al. 2014). In this study, the pH of field soil was around 5.9, which was not the best for the enzyme activity of *Bacillus* spp. The low soil pH in the field was a possible cause of the failures of biologicals in this study. Another possible cause was the disease pressure. Biological agents were found to have good performance in the fields with low disease severity (Larkin 2016; Meng et al. 2012; Raupach and Kloepper 2000). In this study, the disease pressure was high. It was likely to be beyond the capability of biological agents. In other words, the suppressive effect of biologicals was limited when *P. erythroseptica* population was dominant and overwhelming in the soil microbial community. It was also observed in greenhouse trials of this study. In 2015 greenhouse experiment, the inoculum

was so heavy that many plants died and Regalia did not show a significant treatment effect. The inoculum method was adjusted in 2016 greenhouse experiment and all the plants survived, and then the suppressive effects of Regalia on pink rot was observed.

Evaluations on biological products

The performance of biological agents in the field control is not stable (Bale et al. 2008). Many researchers have tried to combine synthetic and biological fungicides, to reduce the use of synthetic fungicides while taking advantage of biological agents in disease control (Elad et al. 1993; Gilardi et al. 2008; Omar et al. 2006). In this study, an enhancing potential of synthetic and biological fungicide combination was found in 2014 trial 1, in which neither oxathiapiprolin (¼ of full rate) nor *Bacillus subtilis var. amyloliquefaciens* Strain FZB24 significantly reduced pink rot disease. However, the combination of them caused a significant reduction in pink rot disease severity, although it was not significantly different from oxathiapiprolin (¼ of full rate) treatment. When fluopicolide was applied at a reduced rate and followed by 4 applications of *Bacillus amyloliquefaciens* F727, the combined treatment significantly reduced pink rot. However, it was hard to separate the effect of fluopicolide from this combination because fluopicolide had a significant suppressive effect on pink rot by itself. These results suggested that combining synthetic and biologicals fungicides could possibly reduce the use of synthetic fungicides and mitigate fungicide resistance problems, although more field trials are needed to confirm the advantages of chemical-biological combinations. The cost effectiveness of

synthetic-biological combined fungicides also needs to be investigated in an economic way, to help growers make better decisions in potato production (Headley 1985; McFayden et al. 2008).

It is also recommended to test different application methods and timings of biological fungicides. Although most biological fungicides available on the market are microbe-based products, there are some deriving from plants. It is necessary to explore the optimum application methods and timings of different biologicals, because they have different MOAs. In greenhouse experiments, MBI110 and Regalia were tested and it was found that early applications (at planting or 24 hours after planting) were better than late applications (after emergence or at tuber initiation phase) in pink rot control. Although in-furrow applications and drenching applications were not compared in the same experiment, Regalia applied through soil drenching had a good performance in 2016, which was not observed in the 2015 Regalia trial where Regalia was applied as a “in-furrow” treatment. Additionally, it was observed that four applications of Regalia (24 h after planting, 10 days after emergence, 20 days after emergence and 40 days after emergence) boosted the health of potato plant underground system and kept mother tubers fresh. This effect was not found in other one-time Regalia treatments in the same experiment. In other words, the application timing and rates of Regalia made a significant difference in pink rot control. Hence, more studies on discovering the optimum application strategies of biologicals are demanded to achieve the full potential of the biologicals.

Integrated pest management (IPM) in pink rot control

Alternative fungicides to mefenoxam and a potential chemical-biological combination were found in this study. Results from this study suggested that the appropriate use of fungicide could mitigate fungicide resistance problems and maximize the efficacy of fungicides. The results of the 2014 field trial 1 showed that reducing the use of mefenoxam and oxathiapiprolin in a field with mefenoxam resistance significantly increased potato tuber yield and slightly reduced potato pink rot severity. In 2015 and 2016 greenhouse trials, multiple applications of Regalia showed an advantage over one-time applications. Therefore, the author developed a hypothesis of “portion control” effect in fungicides. As it is well known, a large portion of foods and beverages leads to substantial increases in energy intake in human bodies, and the energy cannot be efficiently used thus transformed into fat (Rolls 2014). In our case, the question is, with the same total amount, whether smaller amount and multiple applications of a fungicide treatment will have a better performance than a one-time application of the same treatment. It is also worthwhile to study the fungicide retainability of soil and the update fungicide efficiency of plants, and that will help develop the optimum fungicide application strategies.

Conclusions

Synthetic fungicides fluopicolide and oxathiapiprolin-mefenoxam significantly reduced pink rot and increased tuber yield in mefenoxam-resistant fields. Biological fungicides (Taegro, Serenade Soil, Regalia, Double Nickel LC

and MBI-110) did not show any significant treatment effect in the field trials. The combination of chemical and biological fungicides did not show a significant advantage over the sole application of chemical fungicides, yet it suggested a potential to suppress pink rot with reduced rates of chemicals. In greenhouse experiments, Regalia suppressed pink rot, increased tuber yield and enhanced the health of potato underground systems when it was applied multiple times through soil drenching. In sum, experiments in this chapter provided information about the possible alternatives to mefenoxam, the efficacies of biological products and as to how to maximize the effectiveness of fungicides.

Chapter 3

SENSITIVITY AND RESISTANCE RISK OF *PHYTOPHTHORA ERYTHROSEPTICA* TO FLUOPICOLIDE

Chapter Abstract

Thirty-four isolates of wild-type *Phytophthora erythroseptica* were collected from Maine, and examined for their sensitivity to fluopicolide. By measuring the mycelial growth on fluopicolide-amended agar medium, all the isolates were found to be sensitive to fluopicolide, with the effective concentration for 50% inhibition of mycelial growth (EC50) ranging from 0.08 to 0.35 μg/ml. Fluopicolide-resistant mutants were generated from zoospores of 9 out of the 34 wild-type isolates. They were categorized into two types, fast-recovering type and regular type, based on the recover speed on fungicide-free medium. The mycelia of both types of mutants were morphologically abnormal. After the original mutants were transferred to fungicide-free V8 medium consecutively for 10 generations, the 10^{th} generation (T10) of mutants was examined for resistance stability and fitness. The EC50 values of 81.82% of mutants at T10 were significantly higher than those of their wild-type parents, and the fast-recovering type was more tolerant than the regular type originating from the same parent. All the mutants grew significantly slower than their wild-type parents in the first 24 h of incubation at 28 °C, but the growth rate between 24 h and 96 h of mutants, except Mutant 13A14-M, was similar with that of their wild-type parents. All the mutants caused pink rot symptoms on uncut potato tubers, but the virulence of

some mutants was reduced. The results of this study suggested that the risk of *P. erythroseptica* to develop intermediate resistance to fluopicolide was at medium level, and that there was a trade-off between fluopicolide resistance and biological fitness in *P. erythroseptica*.

Introduction

Pink rot (*Phytophthora erythroseptica*) is a ubiquitous potato disease (Porter et al. 2007; Wicks et al. 2000). It causes significant losses in the field and storage (Al-Mughrabi et al. 2007; Mills et al. 2005; Schisler et al. 2009). Currently, few fungicides are effective on potato pink rot (Benson 2009). The systemic chemical fungicide mefenoxam used to be the most effective product (Fitzpatrick-Peabody 2008; Johnson 1997; Wicks 2000). However, the development of mefenoxam-insensitive populations of *P. erythroseptica* has been a big concern in potato production (Goodwin and McGrath 1995; Lambert and Salas 1994; Taylor et al. 2002).

In the field fungicide studies, fluopicolide appeared to be a good alternative to mefenoxam for pink rot control (Zhang et al. 2016). It has good performance in potato fields infested with *P. erythroseptica*, even in the presence of a mefenoxam-resistant *P. erythroseptica* population (see Chapter 2). Fluopicolide is a new oomyceticide with a new mode of action (Toquin et al. 2008). It has been tested on a range of oomycete diseases (Foster 2009; Gevens 2012; Jackson et al. 2010; Matheron 2011; Quesada-Ocampo and Kousik 2015; Rekanovic et al. 2008; Schubert ; Wang et al. 2014a; Wise and

Wilcox 2012). It was reported that some oomycetes have the potential to develop resistance to fluopicolide (Lu et al. 2011; Wang et al. 2014b). Fluopicolide-resistant mutants were selected from the zoospores of wild-type fluopicolide-sensitive *Phytophthora capsica* isolates (Lu et al. 2011). The fluopicolide resistance of *Pseudoperonospora cubensis* was induced by exposing the wild-type isolates to UV light and selecting the adapted *Pseudoperonospora cubensis* isolates on fluopicolide-treated cucumbers (Wang et al. 2014b). However, the risk of fluopicolide resistance in *P. erythroseptica* remains unknown.

Traditional dilution plating method was used in previous studies to test fluopicolide sensitivity of oomycetes (Lu et al. 2011; Wang et al. 2014b). Alternatively, the spiral gradient endpoint (SGE) method can be used in fungicide sensitivity tests (Förster et al. 2004). This method costs less in terms of labor, time and materials. The objectives of this study were: 1) to investigate the baseline sensitivity of *P. erythroseptica* to fluopicolide; 2) to examine the biology and fitness of fluopicolide-resistant isolates; 3) to test the pathogenicity and virulence of the mutants.

Materials & Methods

Pathogen isolates and fluopicolide

Potato tubers showing pink rot symptoms were used for pathogen isolation. Pieces of tubers having partially healthy tissues and partially rot lesions were cut and surface-sterilized in 75% ethanol for 30 s, and then soaked in 0.6% sodium hypochlorite for 5 to 10 min, depending on the necrotic level of tuber

tissue, followed by rinsing three times in sterile distilled water. The disinfested tuber pieces were dried on sterile filter paper and placed on a 1.5% water agar plate. Thirty-Four *P. erythroseptica* isolates were obtained, and purified through the single-spore method. All the isolates were collected from potato fields in Maine, where there was no history of fluopicolide application. Morphological identification (Erwin and Ribeiro 1996), PCR identification with ITS region (White et al. 1990) and mefenoxam sensitivity assay (See Chapter 2) were conducted on the wild-type isolates. The background information of these isolates is listed in Appendix B. Technical grade of fluopicolide (99.1% active ingredient) was obtained from Valent U.S.A. Corporation (1600 Riviera Avenue, Suite 200, Walnut Creek, CA 94596-8025). The chemical powder was dissolved in DMSO, and diluted to a stock concentration ($1x10^5$ µg/ml) for storage and work concentration ($1x10^3$ µg/ml) for later use.

Baseline sensitivity of *P. erythroseptica* to fluopicolide

The baseline sensitivity assay was conducted with a modified spiral plating (based on SGE) method (Förster et al. 2004; Torres-Londono 2016). Pure cultures of *P. erythroseptica* isolates were grown on a clarified V8 plate (Clarified v8 agar: 200 ml clarified V8 juice, 2.5 g CaCO3, 1.5% agar, 800 ml distilled water) with 4 or 8 replicated pieces of sterile cellophane strips per isolate. The cellophane strips had been autoclaved in distilled water for 15 min at 121 °C. The cultures were incubated at 25/28 °C in an incubator, until all the cellophane film strips were covered by mycelia.

An aliquot of 50 ml of potato dextrose agar (PDA) was poured into each 15-mm Petri plate. These PDA plates were left to dry in a sterile hood for an hour. Only the plates with an even surface and no air bubbles were used for spiral plating. An aliquot of 50 µl of fluopicolide ($1x10^3$ µg/ml) was plated on the PDA plates using Spiral Biotech Autoplate 4000 (Spiral Biotech Inc, Norwood, MA, USA) with exponential mode. The plates were left in a sterile hood for 30 minute to ensure fungicide absorbance. Then mycelia-colonized cellophane strips were moved and laid on the fluopicolide-distributed PDA plates, and arranged radially (Figure 3.1). Two or three days later, minimum inhibition concentration (MIC), total inhibition concentration (TIC) and EC50 points were recorded (Fig 3.1). R version 3.2.3 with a modified ECX package (modified based on Torres-Londoño's design) was used to calculate EC50 and analyze EC50 distribution in 34 wild-type isolates (Torres-Londoño et al. 2016).

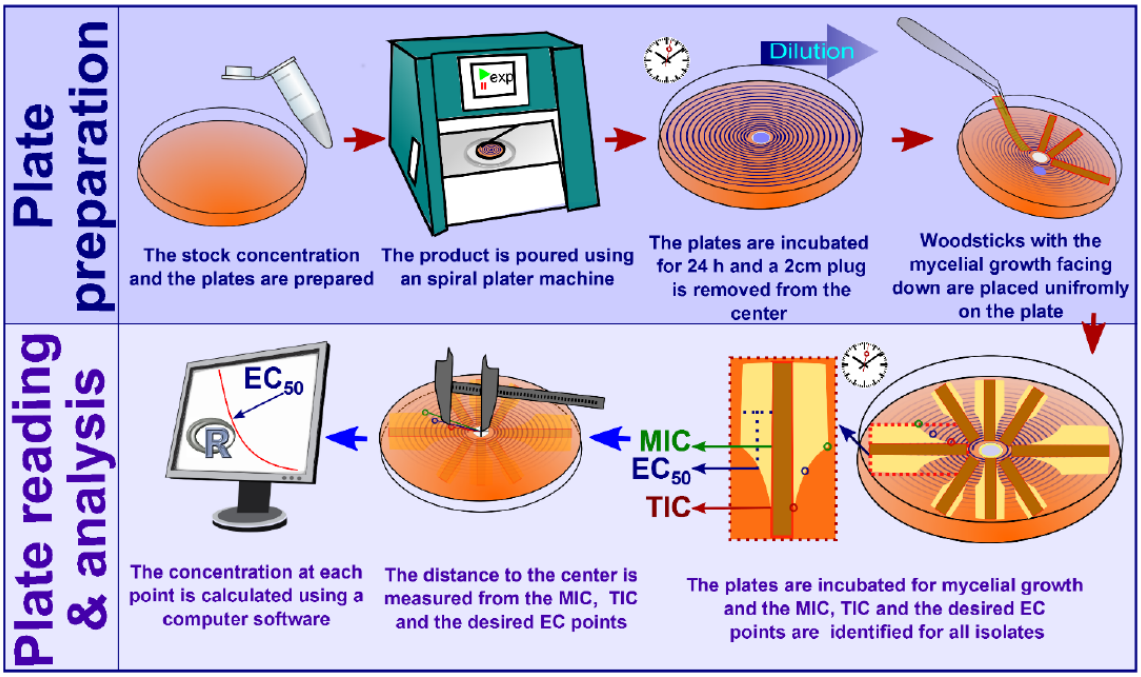

Figure 3.1. Spiral plating procedure (*Torres-Londono 2016*).

Selection of fluopicolide-resistant *P. erythroseptica*

Fluopicolide-resistant *P. erythroseptica* isolates were selected on 100 µg/ml fluopicolide-V8 (F-V8) medium. The V8 juice was filtered to clarify the medium. Zoospores of wild-type *P. erythroseptica* isolates were plated with a sterile hockey rod on F-V8 medium. Zoospores of *P. erythroseptica* were produced following the method published by Al-Mugharbi et al. (Al-Mughrabi et al. 2007), modified by Jiang (unpublished).

To prepare zoospores, *P. erythroseptica* was cultured on 10% V8 agar (room temperature, dark) for 6 days. Four mycelial plugs were punched by a 5-mm cork borer and transferred to a petri plate (10 mm diameter) filled with 10 ml of lima bean broth (LBB). After being incubated at 22 °C under natural light for 3 days, LBB was discarded. The original (10% V8) agar plugs were removed,

and the mycelial mats were rinsed with sterilized distilled water three times. Then the Petri plate (with mycelial mats only) was filled with 10 ml of 10% soil extraction, and incubated at 18 °C under continuous fluorescent light for 4 days (Al-Mughrabi et al. 2007). Four days later, the Petri plate and mycelial mats were rinsed again with sterile water (4 °C, cold water), and 10% soil extraction was replaced by 7 ml of sterile water (4 °C). The plate was chilled at 4 °C for an hour and then left at 22 °C for 30 min. Then the zoospore concentration was estimated with a hemocytometer.

An aliquot of 1 ml of zoospore suspension (1-80 x 10^4 spores/ml) was spread on each F-V8 plate. V8 agar amended with 0.1% DMSO was used as a control and plated with 1 ml of the suspension. After the plates were incubated at 25 °C in the dark for five days, colonies that survived on the F-V8 plates were considered as fluopicolide-resistant mutants (Lu et al. 2011). In this experiment, each isolate had 5 replications, and this experiment was conducted twice. Survival/mutation frequency was calculated: SF/MF = sum of mutants or survivors on five plates/ [(zoospores per plate) x 5].

Morphological observation and resistance stability of fluopicolide-resistant *P. erythroseptica* mutants

The survivors on V8-F plates were transferred to fungicide-free, clarified V8 medium, and the first transfer were considered as the first generation of resistant isolates/mutants (T1). Each transfer counted as a generation. A total of ten generations of resistant isolates were generated and observed under a Leica microscope (Leica Microsystems Inc.,1700 Leider Lane, Buffalo Grove, IL 60089

US) to capture morphological changes. The 10th generation was used to examine the resistance stability of fluopicolide-resistant *P. erythroseptica*. Spiral plating was performed again, as described easier, to test the sensitivity of the 10th generation of mutants and their wild-type parents. This assay was repeated once.

Mycelial growth of *P. erythroseptica* mutants

The 10th generation of *P. erythroseptica* was used to investigate the mycelial growth differences between fluopicolide resistant isolates with their wild-type parents. One agar plug was cut by a 5-mm cork borer and transferred from each active culture of *P. erythroseptica* isolate onto a clarified V8 agar plate. All the tested isolates were incubated at 28 °C, in dark. Each isolate had 4 replications. The radius of mycelial coverage on each plate was measured at 24 h, 48 h, 72 h and 96 h after incubation. Growth curves of each tested isolates were generated based on the serial measurements.

Pathogenicity and virulence of *P. erythroseptica* mutants

Disease-free potato tubers (c.v. Russet Norkotah) were used to examine the pathogenicity and virulence of the isolates. The tubers were washed and disinfested with 0.6% sodium hypochlorite followed by sterile water rinsing. The 10th generation of fluopicolide-resistant *P. erythroseptica* mutants and their wild-type parents on V8 plates were used for inoculation. Three agar plugs of each tested isolate were placed on a random eye of a randomly picked tuber, and covered by a cap of a 2 ml Eppendorf tube. The plastic cap loaded with a test isolate was wrapped and fixed by a piece of Parafilm on the inoculated tuber.

The treated tubers were placed in covered plastic containers, in which, the bottom was covered by a layer of plastic rack. The tubers inoculated with the same mutant/survivor and the ones inoculated with corresponding wild-type parent were incubated in the same plastic container, at the same time at 25 °C. Tubers used as blank controls were treated with sterile agar medium. A completely randomized design with 4 replications was used in this experiment. Tuber lesion penetration was calculated based on Fitzpatrick-Peabody and Lambert's equation: (Depth + Width/2)/2 (Fitzpatrick-Peabody and Lambert 2011). Seven days after inoculation, all the tubers were cut, and the depth and width (using the inoculation site as the center) of symptoms (pink lesion) were recorded.

Statistical analyses

R 3.2.3 and modified EXC package was used in data analysis to calculate EC50 (Torres-Londoño et al. 2016). R 3.2.3 and JMP were used to perform ANOVA and Fisher's LSD (for parametric data) or Wilcoxon each pair analysis (nonparametric data) in fluopicolide sensitivity, mycelial growth (in first 24 h), and virulence comparison. Bivariate analysis was used to examine the linear regression fit of mycelial growth rates between 24 h and 96 h of incubation (Everitt 1995; Matthews et al. 1990). α was set at 0.05 in all the data analyses.

Results

Baseline sensitivity of *P. erythroseptica* to fluopicolide

The spiral plating results showed that all 34 wild-type *P. erythroseptica* isolates were sensitive to fluopicolide (Fig 3.2). The effective concentration for 50% inhibition of mycelial growth (EC50) of those isolates ranged from 0.08 to 0.35 µg/ml, with a mean of 0.18 µg/ml and median of 0.17 µg/ml. Shapiro-Wilk normality test was used to test the distribution of EC50 after log transformation, and the p value was 0.04. ECX (package) results showed the regression coefficient of EC50 calculation was above 0.99. α was set at 0.05 in all the data analyses.

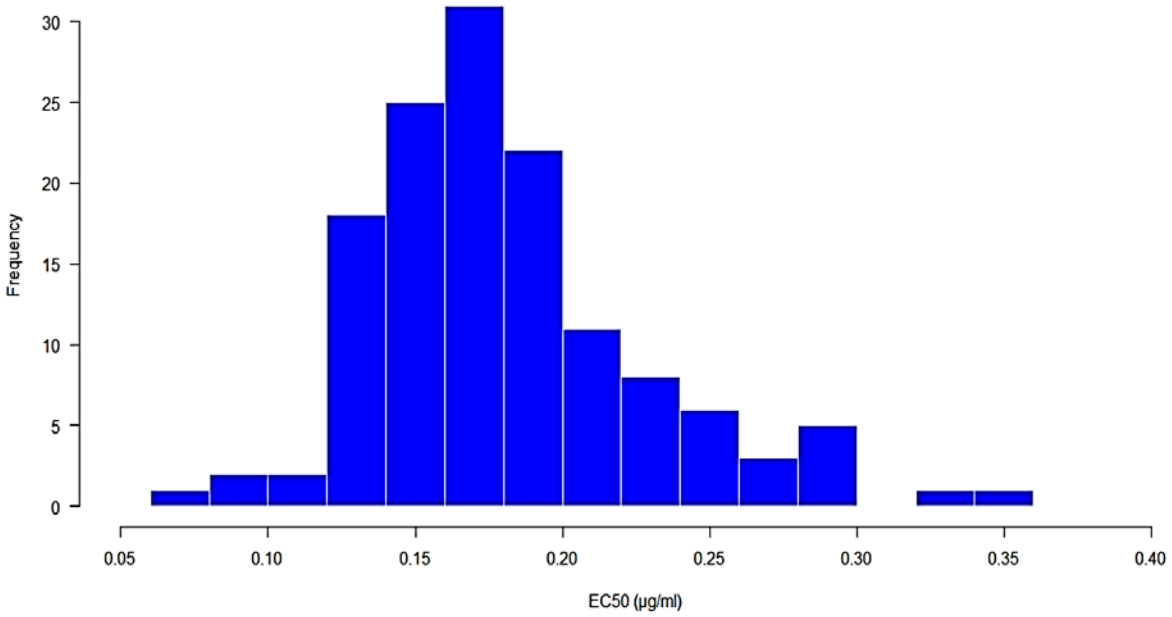

Figure 3.2. Distribution of fluopicolide sensitivity of 34 *Phytophthora erythroseptica* isolates from Maine.

Fluopicolide-resistant *P. erythroseptica* mutants

Among 34 isolates, only 9 produced fluopicolide resistant mutants. The survivors formed visible mycelial colonies on F-V8 plates. The mutation frequency was between 1.35×10^{-5} and 1.00×10^{-4}. A total of 12 mutants were acquired and used for further research, because three wild-type isolates produced two types of mutants with significantly different growth rates. Based on the mefenoxam sensitivity information of wild-type isolates, it seemed that the occurrence of fluopicolide resistance did have a relation with mefenoxam resistance, because both mefenoxam-resistant and mefenoxam-sensitive *P. erythroseptica* can produce fluopicolide-resistant mutants. (See Appendix B).

Morphologies and resistance stability of fluopicolide-resistant *P. erythroseptica* mutants

In total, ten generations of resistant isolates were acquired. Each generation (including the original one) was monitored with a Leica-microscope to capture the morphological changes. Images of the germination of original mutants were captured: the survived zoospores germinated thick and swollen hyphae (Fig 3.3). The first generation (T1) was obtained after transferring the original mutants to clarified V8 plates. It was noticed that the growth rates of some mutants were significantly different from the others. The survivors, the mutants, started to recovered on V8 plates. However, mutants at T1 formed short branched, swollen septa-free hyphae (Fig 3. 4), which was different from typical *Phytophthora erythroseptica* hyphae and other oomycetes. Some mutants

eliminated malformed mycelial at T2, but some did not recover from it until T3 or T4. Some mutants did not produce oospores between T3 and T6, but the oospore reproduction was back to normal in all the mutants at T8. One mutant stopped producing oospores at T5 and eventually died at T6. Multiple attempts to transfer it from T4 and T5 failed.

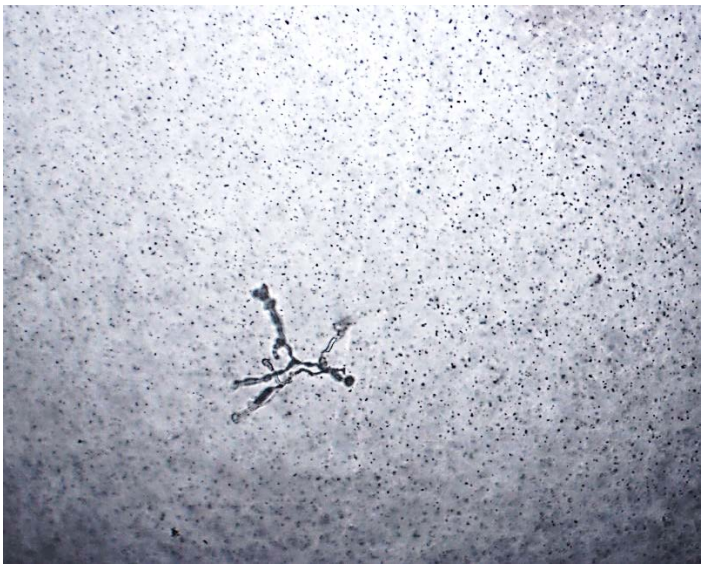

Figure 3.3. Germination of a fluopicolide-resistant zoospore of *Phytophthora erythroseptica*

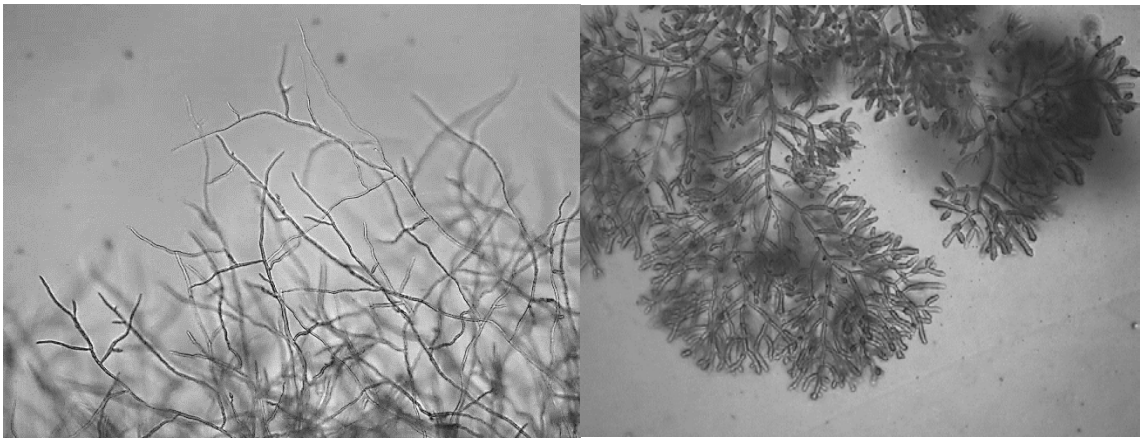

Figure 3.4. Mycelia formed by wild-type isolates (left) and T1 mutants (right) of *Phytophthora erythroseptica*.

Spiral plating assay was conducted using the 10th generation (T10) and the wild-type parents of all the mutants. The results showed that the EC50 values of wild-type isolates generated in this assay were similar with the ones generated in the baseline sensitivity test (Table 3.1, $\alpha = 0.05$). It demonstrated that the spiral plating method was reliable. The data collected from Isolate 13A02, Isolate 13A05, Isolate 13A08 and Isolate 13A14 were nonparametric data. Therefore, Wilcoxon each pair test was used to separate their EC50 mean values. The rest was analyzed through ANOVA and Fisher's LSD. Data analysis results showed the EC50 values of mutants at the 10th generation were significantly different with those of their parents, except 13A03-M and 13A08-M. The fast-recovering (fast-growing at T1) type of mutants were more resistant/tolerant to fluopicolide than the regular type from the same parents.

Table 3.1. Effective concentration of 50% inhibition (EC50, µg/ml) of mycelial growth of fluopicolide resistant mutants and their wild-type parents of *Phytophthora erythroseptica*

	13A01	13A02	13A03	13A05	13A06	13A07	13A08	13A14	13A39
WT'[a]	0.18 a[b]	0.18 a	0.25 a	0.17 a	0.22 a	0.20 a	0.21 a	0.22 a	0.21 a
WT	0.21 a	0.16 a	0.21 a	0.18 a	0.22 a	0.25 a	0.24 a	0.20 a	0.18 a
MT1	0.33 b	0.24 b	0.25 a	0.47 b	0.94 b	0.41 b	0.39 a	> 5.31 b	0.44 b
MT2[d]	N/A	0.99 c	N/A	1.29 c	N/A	N/A	N/A	N/A	N/A

[a] WT' indicates the EC50 of wild-type isolates that were generated in baseline sensitivity test. WT indicates the EC50 of wild-type isolates that were tested with their mutants on the same spiral gradient F-V8 plates.
[b] Mean values not connected by the same letters are significantly different (α=0.05).
[c] The mycelial growth of mutants originating from 13A14 was not suppressed on the spiral gradient F-V8 plates (the highest concentration was 5.31 µg/ml).
[d] Isolate 13A02 and isolate 13A05 produced two types of mutants that had significantly different grow rates at T1. MT1 indicates the type of mutants having a regular grow rate. MT2 indicates the fast-growing type.

Mycelial growth rate of fluopicolide-resistant *P. erythroseptica* mutants

The radius of mycelial growth on each plate was measured at 24 h, 48 h, 72 h and 96 h of incubation. The original agar plug radius (0.25 °Cm) was subtracted before data analysis. Mycelial growth data collected at 24 h were used to compare the growth difference between mutants and wild-type parents in the first 24 hours of incubation. The results showed that mutants grew significantly slower than wild-type parents in the first 24 hours (Table 3.2).

Table 3.2. Growth rate (cm/h) of mutants and wild-type parents of *Phytophthora erythroseptica* measured at 24 h of incubation.

	Isolate								
Type[a]	13A01	13A02	13A03	13A05	13A06	13A07	13A08	13A14	13A39
WT	0.83 a[b]	0.96 a	0.88 a	0.85 a	0.75 a	0.81 a	0.88 a	0.82 a	0.91 a
MT1	0.77 b	0.71 b	0.71 b	0.64 b	0.60 b	0.59 b	0.61 b	0.20 b	0.75 b
MT2	N/A	0.53 c	N/A	0.38 c	N/A	N/A	N/A	N/A	N/A

[a] WT: wild-type; MT: mutant.
[b] Mean values not connected by the same letters are significantly different (α = 0.05).

Mycelial growth data collected at 24, 48, 72 and 96 h of incubation were used for linear regression analysis. The results showed that R^2 ranged from 0.9045 to 0.9985, which suggested the growth data of each tested isolate fit the linear regression model very well. The growth rates between 24 h and 96 h were indicated by the slopes of each regression equation. The results showed the growth rates of mutants and their parents between 24 h and 96 h were close (Table 3.3). However, the growth rate of Mutant 13A14-M was lower than its wild-type parent.

Table 3.3. Growth rate (24 to 96 h incubation) of mutants and wild-type parents of *Phytophthora erythroseptica* analyzed by linear regression equation.

Isolate	Growth rate[a] (cm/h)	R Square
13A01	0.0297	0.9954
13A01-M	0.0315	0.9955
13A02	0.0283	0.9972
13A02-M1	0.0268	0.9971
13A02-M2	0.0297	0.9945
13A03	0.0303	0.9984
13A03-M	0.0303	0.9984
13A05	0.0322	0.9860
13A05-M1	0.0301	0.9045
13A05-M2	0.0291	0.9985
13A06	0.0329	0.9975
13A06-M	0.0283	0.9913
13A07	0.0295	0.9929
13A07-M	0.0295	0.9929
13A08	0.0304	0.9942
13A08-M	0.0260	0.9959
13A14	0.0315	0.9981
13A14-M	0.0226	0.9933
13A39	0.0288	0.9899
13A39-M	0.0296	0.9967

[a] Growth rate is the slope of regression equation generated by bivariate analysis.

Pathogenicity of fluopicolide-resistant *P. erythroseptica* mutants

All the of fluopicolide-resistant mutants showed pathogenicity on uncut potato tubers with a pink rot incidence of 100%, except that Mutant 13A14-M only caused 25% pink rot (1 out of 4 tubers was infected) in the first tuber inoculation experiment. The experiment was repeated, and the data were consistent. Data were analyzed through ANOVA or student t test. Mean values were compared within the group sharing the same origin background. P value less than 0.05

indicates a significant difference. The results showed the virulence of Mutant 13A14-M was much lower than its wild-type parent and other mutants (Table 3.4).

Table 3.4 Lesion development on potato tubers inoculated by *Phytophthora erythroseptica* mutants and wild-type parents

Isolate[a]	Depth (cm)	*P* value[b]	Width (cm)	*P* value	Penetration (cm)	*P* value
13A01	3.95		6.55		3.61	
13A01-M	3.90	0.90 NS	7.18	0.29 NS	3.74	0.64 NS
13A02	4.00		7.83		3.96	
13A02-M1	3.78		6.55		3.53	
13A02-M2	3.23	0.31 NS	5.78	0.31 NS	3.06	0.28 NS
13A03	3.58		7.73		3.72	
13A03-M	3.58	1.00 NS	6.88	0.22 NS	3.51	0.48 NS
13A05	3.35		5.90		3.15	
13A05-M1	3.93	0.23 NS	6.50	0.21 NS	3.59	0.18 NS
13A05-M2	2.73		4.78		2.56	
13A06	3.88		7.83		3.89	
13A06-M	3.03	0.17 NS	5.88	0.03	2.98	0.02
13A07	3.50		6.23		3.31	
13A07-M	3.60	0.72 NS	7.20	0.06 NS	3.60	0.03
13A08	3.95		7.30		3.87	
13A08-M	2.98	0.09	5.98	0.09 NS	2.06	0.06 NS
13A14	4.17		7.33		3.18	
13A14-M	2.00	<0.001	5.00	<0.001	3.23	<0.001
13A39	4.23		6.63		3.78	
13A39-M	4.10	0.43 NS	6.75	0.35 NS	3.74	0.39 NS

[a] Isolates with the suffix “M” indicate mutants derived from the same isolate.
[b] $P < 0.05$ indicates a significant difference. NS: not significantly different.

Discussion

All of Maine isolates of *P. erythroseptica* were sensitive to fluopicolide. This result was not surprising as fluopicolide has not been broadly used in Maine, and the pathogen did not have enough selection pressure. This is similar to the observations of *Phytophthora capsici* populations in Michigan and *Pseudoperonospora cubensis* populations in China (Lu et al. 2011; Wang et al. 2014b). However, this does not mean that there is no risk of resistance development.

It is likely there is variation in *P. erythroseptica* 's response to fluopicolide. The mutant selection experiment was conducted twice. The mutants were derived from 9 of 34 wild-type *P. erythroseptica* isolates, while the rest of the wild-type isolates did not produce any mutants. The estimated mutation frequency in this study was approximately 1×10^{-5}, which was higher than previously reported fluopicolide resistance mutation frequency in other oomycete species (Lu et al. 2011; Wang et al. 2014b). This result suggests a medium risk of fluopicolide resistance in *P. erythroseptica* populations (Brent and Hollomon 2007a).

In this study, the morphological change of fluopicolide-resistant *P. erythroseptica* mutants was observed. It was observed that fluopicolide in the F-V8 selection medium caused the misshapen hyphae in *P. erythroseptica* mutants. Fluopicolide could also influence the oospore reproduction in resistant mutants (morphological observation under light microscope, data not shown). Some mutants stopped producing oospores on V8 plates from T3 (plates were

kept for 3-4 weeks for oospore observation). One of them died at T6, and the rest resumed oospore reproduction from T7. Currently, fluopicolide is known for acting through inhibiting spectrin-like proteins (Toquin et al. 2008; Toquin et al. 2010). It could explain why the mycelium was damagedby a lack of the support of proteins. However, there was no studying showing the influence of fluopicolide on oospores. Further studies are required to investigate this matter.

A trade-off between a resistance development and biological fitness may occur in plants, bacteria and fungi (Brown and Rant 2013; Damicone and Smith 2009; Hall et al. 2004; Hobbelen et al. 2014; Kang and Park 2010; Mikaberidze et al. 2014; Montarry et al. 2007). For example, Kang and Park (2010) showed that there was a fitness cost to gain antibiotic resistance in bacteria, Acinetobacter sp. Fluopicolide-resistant *Pseudoperonospora cubensis* mutants showed differences with their wild-type parents in latent period, infection frequency, lesion extension and sporulation ability (Wang et al. 2014b).However, there was no difference between most fluopicolide-resistant *Phytophthora capsici* mutants and their wild-type parents in zoospore production, cyst germination and virulence (Lu et al. 2011).

The trade-off was observed in this study. Although the mutants were capable of infecting potato tubers, the virulence of mutants was generally weaker than wild-type parents. The fast-recovering type mutants seemed to be more tolerant to fluopicolide. But the virulence of them, like 13A02M2, can be compromised. Mutant 13A14-M seemed to be a highly resistant, but its virulence

was weakened. It only caused 25% disease incidence in the first pathogenicity test.

Conclusions

All Maine isolates of *P. erythroseptica* collected in this study were sensitive to fluopicolide, but there was a potential risk of developing pathogen population resistant to fluopicolide. Nine wild-type isolates produced fluopicolide-resistant mutants. The mycelial morphology of fluopicolide-resistant mutants was abnormal. The EC50 values of 81.82% of mutants at T10 were significantly higher than those of their wild-type parents, and the fast-recovering type of mutants was more tolerant than the regular type originating from the same parent. However, the fitness of some mutants was impaired, resulting in reduced mycelial growth, unstable oospore reproduction and reduced aggressiveness in pathogenesis. The predicted risk of fluopicolide resistance in *P. erythroseptica* was at a medium level. It was likely that there was a trade-off between fluopicolide resistance and biological fitness in some *P. erythroseptica* isolates.

Chapter 4

EFFECTS OF CROP ROTATION ON PINK ROT OF POTATO

Chapter Abstract

A two-year crop rotation experiment was conducted to determine the effects of rotation crops on potato pink rot. Two fields (A and B) were initiated by inoculating the soil with *Phytophthora erythroseptica* inoculum before planting in 2014, followed by different cropping arrangements: Field A was planted with potato plants in 2014, and rotated with either alfalfa, barley-ryegrass, canola, red clover, pumpkin, sweet corn, oats or potatoes in 2015. Field B was planted with alfalfa, barley-ryegrass, canola, red clover, onion, sweet corn, oats or potatoes in 2014, and rotated with potatoes in 2015. In 2016, Field A was planted with potatoes. Disease and potato tuber yield were assessed at harvest. The results showed that alfalfa, canola and pumpkin significantly increased potato tuber yield. However, the rotation crops had no significant effect on pink rot.

Introduction

Potato pink rot is a ubiquitous soil-borne disease caused by an oomycete pathogen, *Phytophthora erythroseptica* (Cairns and Muskett 1933; Gudmestad et al. 2007; Taylor et al. 2004). It causes significant losses in the field and storage (Rai 1979; Toms 1968; Yellareddygari et al. 2016). Currently, the pink rot management mainly relies on fungicide control, and the systemic chemical

mefenoxam has been the most effective fungicide for pink rot control. However, the mefenoxam resistance of *P. erythroseptica* has been a problem in many states including Maine, New York, Idaho, Michigan, Nebraska, Philadelphia and North Carolina (Goodwin and McGrath 1995; Lambert and Salas 1994; Porter et al. 2007; Taylor et al. 2002; Venkataramana et al. 2010). New fungicides and new management strategies are needed to control potato pink rot.

Crop rotation has a long history in agriculture, and it is known for its capability of improving crop quality and yield (Honeycutt et al. 1996; Honeycutt 1998; Johnson and Cummings 2015). By growing different types of crops between seasons, it replenishes soil nutrient resources, improves soil properties, and reduces erosion (Larkin et al. 2010). With its impacts on the soil microbial communities, crop rotation breaks up the life cycle of soilborne plant pathogens, and changes the abundance and diversity of the soil microbiome that favors plant health (Larkin et al. 2010; Sudini et al. 2011). Crop rotation has been one of the practical options in controlling soilborne diseases of potato (Larkin 2008; Larkin et al. 2010; Peters 2003; Toquin et al. 2008). It does not only suppress soilborne diseases directly (Larkin 2010), but also confers disease-resistance to potatoes from pathogen (*P. erythroseptica*) attack (Peters et al. 2005a).

Different crops can have dramatically different effects on crop diseases (Mazzola 1999). For instance, canola (*Brassica napus*), and rapeseed (*Brassica napus*) consistently reduce the severity of Rhizoctonia canker, black scurf, and common scab (Larkin et al. 2010) on potatoes; Sweet clover (*Melilotus officinalis*) and hay can suppress on Verticiilium wilt (Emmond 1972). However, so far, there

have not been any studies revealing crop rotation effects on pink rot and the corresponding correlation between cropping sequences and soil microbial community structure and activity.

Some rotation crops produce antimicrobial compounds that directly inhibit or reduce a wide range of bacteria and fungi including soilborne pathogens on potato (Ojaghian 2012). The *Brassica* family (e.g. canola, rapeseed) has the capability to produce glucosinolates, which will become isothiocyanates after further reaction (Ojaghian 2012). Isothiocyanates are known as biofumugants that have a suppressive effect on various soil organisms (Larkin 2007; Mazzola 2005; Ojaghian 2012). Other than glucosinolates, there might be some additional substances released by *Brassica* crops that can also suppress fungal pathogens (Mazzola 2005). The *Allium* family (e.g. garlic, onion) is another group that is capable of releasing antifungal and antibacterial chemicals. Researchers found that *Allium* family can release diallyl sulfide and allicin, which are both noted for their antimicrobial activity (Benkeblia 2007). It was also reported that PGPR (Plant growth promoting rhizobacteria) bacteria, *Rhizobium leguminosarum* bv. trifolii, is associated with clover (*Trifolium pratense*) and wheat (*Triticum spp.*.) roots (Urban 1982). It is reasonable to hypothesize that the growth of these crops can affect the population of *Phytophthora erythroseptica* in soil, which would make these crops good crop rotation choices to control potato pink rot.

The objective of this study was to observe the rotation effects of alfalfa, barley/ryegrass, canola, red clover, onion, pumpkin, sweet corn and oats on potato tuber yield and pink rot incidence and severity.

Materials & Methods

Isolates of *P. erythroseptica* used in field trials

Seven *P. erythroseptica* isolates were collected: four were from a UMaine collection (Fitzpatrick-Peabody and Lambert 2011) and three were isolated from diseased tubers found in uninoculated fields at Aroostook Farm, Presque Isle, Maine. The pathogens were isolated from potato tubers showing pink rot symptoms. Pieces of tuber tissue were cut and surface-sterilized in 75% ethanol for 30s, and then soaked in 0.6% sodium hypochlorite for 5 min to 10 min (depending on the necrotic level of tuber tissue). After being rinsed three times in sterile distilled water, three tuber pieces were dried on a sterile filter paper and placed on a 1.5% water agar (15 g of agar powder: 1000 ml of water) plate. The pathogens isolated from the same tuber were considered to be different copies of the same isolate. All the isolates were identified through PCR and Sanger sequencing. DNAs were extracted and amplified with ITS1/ITS4 primers (White et al. 1990). The amplicons were sequenced and compared with *P. erythroseptica* sequences in NCBI database (Wheeler et al. 2007). The similarity was above 99%. Three isolates from this collection (two mefenoxam-sensitive isolates and one mefenoxam-resistant isolate) were used in field inoculum preparation (See Chapter 2).

Preparation of *P. erythroseptica* inoculum and field inoculations

Using a modified method based on Erwin's (Erwin and Ribeiro 1996), culture bags were made with 21" x10" x 5" mushroom spawn bags (Out Grow) containing 6 L of vermiculite (medium size) and 2.1 L of V8 broth (consisting of 0.6 L of V8 juice, 1.5 L of water and 7.5 g of calcium carbonate). The bags were autoclaved twice in 48 hours with a liquid 45min cycle (Amsco Lab 250 laboratory steam sterilizer). Cultures of *P. erythroseptica* were transferred to the culture bags and incubated at room temperature. During the inoculum growth stage, the moisture in the bags were monitored and adjusted to be optimum to *P. erythroseptica*. In the meantime, the mycelial growth of field inoculum was examined weekly. The concentration of oospores was monitored in late stages (*P. erythroseptica* maturing stage). The culture bags were shaken periodically, to distribute nutrients and oxygen evenly and make them available, thus producing better mycelia extension and oospore growth. The vermiculite-based inoculum was used in field trials after four to six weeks of incubation.

Experimental design of field trials and plot maintenance

Two adjacent fields (46°39'N, 68°01'W), designated as A and B, were assigned at Aroostook Farm, Presque Isle, Maine. Each field had 48 rows with 104 feet in length. Fertilizer (14-14-14 NPK, Cavendish) at 1,110 lb/a and insecticide (Admire, Bayer) at 8.7 oz/a were applied in furrow at planting. Inoculum of *P. erythroseptica* was evenly spread in the furrow before planting. Four rows in the middle of each plot were inoculated, with 2.25 L of vermiculite

inoculum per row. The furrows were covered soon after planting to ensure the survival of pathogens.

A randomized complete block design was used in this experiment. Each field contained 4 blocks, with an 8-foot gap between each. In each block, there were 8 plots, which contained six 20-feet rows. The row spacing was 3 feet, and the plant spacing was 1 foot. Eight treatments: alfalfa (*Medicago sativa*), barley (*Hordeum vulgare*) undersown with ryegrass (*Lolium multiflorum*), canola (*Brassica napus*), red clover (*Trifolium pratense*), onion or pumpkin (*Allium cepa/Cucurbita pepo*), sweet corn (*Zea mays*), Potato *(Solanum tuberosum*) and oats (*Avena sativa*) were randomly assigned to the plots in each block. Potato *(Solanum tuberosum*, cv. *Russet Norkotah*) was used as control.

The field A was arranged with the following sequences of crops: potato in 2014, rotation crops [alfalfa, barley (undersown with ryegrass), canola, clover, onion, sweet corn, oats and potato] in 2015, and potato in 2016. Field B was in rotation crops [alfalfa, barley/ryegrass, canola, clover, pumpkin (replacing onion), sweet corn, oats and potato] in 2014, and potato in 2015. During the growing seasons, weather conditions in Presque Isle were monitored, and hourly weather update were acquired at the website (http://www.umaine.edu/umext/potatoprogram/Current_Vantage_Pro.htm). Weeds were removed weekly, and late blight were controlled by fungicide Bravo ZN (1.5 qt/a, Syngenta, Greensboro, NC 27419) as described in Chapter 2.

Plant emergence

In 2015, potato plant emergence was recorded six weeks after planting. Emerged plants of the two middle rows of each plot were counted. Plant emergence was calculated based on the percentage of emerged seeds out of total number of seed tubers in that row (Eq.2.1.). Plant emergence was not recorded in 2016.

Assessment of pink rot disease and potato tuber yield

At harvest, tubers from the middle 2 rows were collected using a one-row digger. The tubers were cleaned with washing equipment and weighed for yield. In 2015, all the tubers were weighed to collect yield data, but only one row was used for disease evaluation. In 2016, only one middle row was for data collection. Pink rot incidence was the percentage of infected tubers in harvested tubers. Pink rot severity was rated by reading the approximate coverage of the visible symptom (pink staining) on the cut surface of tubers, based on a scale of 0 = no symptoms, 1 = < 2.5% of surface with symptoms, 2 = 2.5 – 10% surface with symptoms, 3 => 10 – 25% surface with symptoms, 4 => 25–50% surface with symptoms and 5 => 50% surface with symptoms of susceptibility. Disease index was calculated using Eq.2.2. (Chapter 2) to express disease severity. Harvested tubers were also examined for other soilborne diseases, including common scab and black scurf.

Data analysis

The yield and disease severity data were analyzed using JMP 10. (SAS Institute, Cary, North Carolina). The means values were compared using

nonparametric test with Wilcoxon each pair comparison, or ANOVA with Fisher's LSD, at $\alpha = 0.05$.

Results

Plant emergence

In 2015, Field A was under rotation and Field B was covered by potatoes. Four plots treated with potatoes in Field A were examined for emergence. The average of emergence was 87.50 (%), and the standard deviation was 4.33. Plant emergence data were collected from all the plots of Field B. ANOVA showed that rotation crops did not affect the emergence of potato plants in Field B (Table 4.1). Emergence data of rotation trails in 2016 were not recorded.

Table 4.1. Potato plant emergence in Field B, 2015.

Treatment	Emergence of potato (%)[a]
Alfalfa	80.6 a
Barley & Ryegrass	80.6 a
Potato	80.6 a
Oats	80.0 a
Clover	79.4 a
Corn	78.8 a
Onion	76.3 a
Canola	73.8 a

[a] Means values followed by the same letters are not significantly different (α = 0.05).

Assessment of pink rot disease and potato tuber yield

In 2015, potato tubers in middle two rows in each plot were harvested from Field B. Tuber yield, the disease incidence and severity of pink rot and other soilborne diseases were assessed. Data analysis showed that alfalfa significantly increased tuber yield in comparison with prior rotation with potatoes, although none of the rotation crops had a significant effect on pink rot incidence or severity (Table 4.2.). Black scurf and common scab were found on harvested tubers. Statistical analysis indicated Alfalfa significantly increased the disease incidence and severity of common scab (Table 4.3.). The rotation crops did not have a significant effect on black scurf disease.

Table 4.2. Potato tuber yield, pink rot severity and pink rot incidence (after rotation) in Field B, 2015.

Treatment	Total yield (lb/plot)	Pink rot incidence (%)	Pink rot severity (%)
Alfalfa	38.07 a[a]	4.71 a	4.75 a
Clover	35.98 ab	2.21 a	2.25 a
Onion	35.13 ab	2.74 a	2.75 a
Corn	31.44 ab	2.72 a	2.75 a
Canola	27.12 ab	5.09 a	5.25 a
Barley & Ryegrass	27.03 ab	1.21 a	1.25 a
Oats	26.46 ab	0.35 a	0.25 a
Potato	24.00 b	5.51 a	5.75 a

[a] Mean values not connected by the same letter are significantly different (α = 0.05).

Table 4.3. Incidence and severity of other soilborne diseases on potato (after rotation) in Field B, 2015.

Treatment	Black Scurf incidence (%)	Black Scurf severity (%)	Common Scab incidence (%)	Common Scab severity (%)
Alfalfa	18.89 a[a]	10.88 a	78.76 a	40.15 a
Clover	15.57 a	10.42 a	50.37 b	26.12 b
Onion	19.07 a	11.89 a	50.06 b	23.34 b
Corn	15.66 a	9.59 a	48.36 b	21.25 b
Canola	17.69 a	9.55 a	48.36 b	24.42 b
Barley & Ryegrass	23.05 a	14.02 a	53.16 b	25.25 b
Oats	21.45 a	11.04 a	46.77 b	19.41 b
Potato	30.87 a	19.96 a	52.83 b	26.38 b

[a] Mean values not connected by the same letter are significantly different (α = 0.05).

Data analysis showed that canola, pumpkin and alfalfa significantly increased tuber yield in comparison with potatoes, although none of the rotation crops had a significant effect on pink rot incidence or severity (Table 4.4). Black scurf and common scab were found on harvested tubers. Statistical analysis showed that the rotation crops did not have any significant effects on black scurf or common scab disease (Table 4.5.).

Table 4.4. Potato tuber yield, pink rot severity and pink rot incidence (after rotation) in Field A, 2016.

Treatment	Total yield (lb/plot)	Pink rot incidence (%)	Pink rot severity (%)
Canola	17.88 a[a]	6.31 a	5.66 a
Pumpkin	17.33 a	9.42 a	7.72 a
Alfalfa	17.03 a	10.90 a	9.64 a
Oats	15.98 ab	5.67 a	5.23 a
Barley & Ryegrass	15.91 ab	7.32 a	6.18 a
Corn	15.08 ab	7.35 a	7.25 a
Clover	13.95 ab	4.23 a	4.23 a
Potato	10.77 b	10.36 a	10.35 a

[a] Mean values not connected by the same letter are significantly different (α = 0.05).

Table 4.5. Incidence and severity of other soilborne diseases on potato (after rotation) in Field A, 2016.

Treatment	Black scurf incidence (%)	Black scurf severity (%)	Common scab incidence (%)	Common scab Severity (%)
Alfalfa	30.44 a[a]	12.46 a	68.47 a	29.05 a
Clover	52.93 a	20.25 a	60.22 a	26.12 a
Pumpkin	38.18 a	17.93 a	61.70 a	29.00 a
Corn	52.30 a	22.11 a	66.49 a	21.25 a
Canola	43.38 a	13.32 a	63.30 a	25.73 a
Barley & Ryegrass	43.25 a	22.07 a	63.89 a	28.69 a
Oats	50.02 a	22.85 a	56.74 a	23.26 a
Potato	42.31 a	25.91 a	64.93 a	29.63 a

[a] Mean values not connected by the same letter are significantly different (α = 0.05).

Discussion & Conclusions

Planting alfalfa prior to potato significantly increased potato tuber yield in the subsequent season. Rotating canola and pumpkin with potatoes significantly increased potato tuber yield in Field A. In Field B (2015), alfalfa significantly increased potato common scab, but it did not have significant effects on pink rot or black scurf. In Field A, there was no treatment effects on pink rot, black scurf or common scab.

The results in this study have demonstrated that rotating potato with alfalfa, pumpkin and canola significantly increased tuber yield. Unfortunately, pink rot was not significantly affected by any of the rotation crops. Many studies

showed that the effects of 2-year rotation on potato diseases, tuber quality and tuber yield are limited (Johnson and Cummings 2015; Larkin et al. 2010; Myers et al. 2008; Peters et al. 2005a). Longer rotations are highly recommended by researchers to achieve better disease management and higher revenue (Johnson and Cummings 2015; Larkin et al. 2010; Myers et al. 2008). In this study, none of tested rotation crops suppressed potato soilborne diseases, which was similar with the results from other potato rotation studies. However, it was possible that some rotation crop did suppress soilborne pathogens or change the soil microbial pattern in a beneficial way, although the change was not sufficient to cause a significant reduction in soilborne disease severity. It is anticipated that the result of Illumina sequencing data analysis could reveal the changes of soil microbial patterns in different rotation treatment (see Appendix C).

Alfalfa in both two-year rotation (potato-alfalfa-potato and alfalfa-potato) fields significantly increased potato tuber yield. Alfalfa belongs to the legume family, and it is known for its symbiotic relationship with nitrogen-fixing bacteria (Bruulsema and Christie 1987; Hesterman et al. 1986; MacKenzie et al. 1997; Voss and Shrader 1984). Therefore, the increase of tuber yield could be associated with the increases nitrogen fixation benefits brought by alfalfa and nitrogen-fixing bacteria. This hypothesis could be tested by analyzing the Illumina sequencing data of soil microbial DNAs collected from different treatments.

Canola improved tuber yield in Field A (2016), but this benefit was not found in Field B (2015). Therefore, the effect of canola was inconclusive. Onion was used in Field B (2014). However, the establishment and growth of onion

plants was not good due to the dry weather. Therefore, pumpkin was used in Field A (2015). Although the result in Field A (2016) showed that pumpkin significantly increased potato tuber yield, a repeated experiment is required to confirm the effect of pumpkin on potato tuber yield.

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APPENDICES

APPENDIX A. RECIPES OF MEDIUM USED IN ZOOSPORE REPRODUCTION

10% V8 agar:

20 ml V8 juice, 0.25 g Calcium Carbonate, 12 g agar, 980 ml water.

Lima bean broth (LBB):

Add 30 g lima bean seeds in a 2-L flask filled with 600 ml water. After autoclaving, filter the broth through 4 layers of cheese cloth to remove the bean residuals. Then add up to 1 L with sterile water and autoclaved again.

10% soil extraction:

Add 100 g soil into a 2-L flask filled with 1 L water. Stir for 30 min and store at 23°C overnight. Transfer the supernatant to a clean 1-L flask, and add up to 1 L with sterile water and autoclaved again.

APPENDIX B. PHYTOPHTHORA ERYTHROSEPTICA ISOLATES

Table B.1 Isolates of *Phytophthora erythroseptica* used in this study

Isolate N.O.	Sensitivity to mefenoxam	Source
13A01	Sensitive	H. Jiang
13A02	Sensitive	H. Jiang
13A03	Sensitive	H. Jiang
13A04	Resistant	H. Jiang
13A05	Resistant	H. Jiang
13A06	Sensitive	H. Jiang
13A07	Resistant	H. Jiang
13A08	Resistant	H. Jiang
13A09	Sensitive	H. Jiang
13A10	Resistant	H. Jiang
13A12	Resistant	H. Jiang
13A13	Resistant	H. Jiang
13A14	N/A	H. Jiang
13A39	Resistant	W. Mu
13A40	Resistant	W. Mu
14B71	N/A	X.Y. Zhang
14B72	N/A	X.Y. Zhang
16A01	Sensitive	D. Lambert
16A02	Sensitive	D. Lambert
16A03	Sensitive	D. Lambert
16A04	Sensitive	D. Lambert
16A05	Sensitive	D. Lambert
16A06	Sensitive	D. Lambert
16A07	Sensitive	D. Lambert
16A08	Sensitive	D. Lambert
16A09	Resistant	D. Lambert
16A10	Resistant	D. Lambert
16A11	Resistant	D. Lambert
16A12	Sensitive	X. M. Zhang
16A13	Sensitive	X. M. Zhang
16A14	Sensitive	X. M. Zhang
16A15	N/A	X. M. Zhang
16A16	N/A	X. M. Zhang
16A17	N/A	X. M. Zhang

APPENDIX C. EFFECTS OF CROP ROTATION ON SOIL MICROBIAL COMMUNITIES

Introduction

Since soil microbiota responds quickly to environmental changes (Meriles 2009), it is hypothesized that the effects of rotation crops on the characteristics and structure of soil microbial communities can be revealed by comparing soil microbial community structure and population at different stages: before and after rotation. Soil microbial communities, primarily bacterial communities, have been analyzed using metagenomics through next generation sequencing by analyzing the amplicons from critical regions in fungal and bacterial genome (Caporaso et al. 2012; Rosenzweig et al. 2012; Sugiyama et al. 2010; Sul 2009). The bacterial, fungal and oomycete communities in soil have already been successfully analyzed on the Illumina platform (Kozich et al. 2013; Reed and Mazzola 2015; Sapkota and Nicolaisen 2015; Schmidt et al. 2013). Therefore, the soil microbial communities in potato fields can be investigated using Illumina sequencing.

The development of integrated pink rot management requires the understanding of pink rot disease associated soil microbial community. It is necessary to investigate the impact of different crops on pink rot disease and the influence of crops on soil microbes to shed light on crop rotation and biological controls in potato pink rot management. The emergence and development of next generation sequencing (NGS) enables researchers to fulfill the investigation

on soil microbial community associated with pink rot. The objective of this study was to investigate the soil microbial patterns associated with different rotation crops and different levels of pink rot disease.

Materials & Methods

Soil sampling and DNA extraction

During each season, soil was sampled near the roots of plants at planting and at harvest, using the methods described by Larkin et al (2010). Five soil cores were taken using a soil probe from the middle 2 rows in each plot, with the depth of 8 to 15 cm and the diameter of 2 cm. At sampling, five soil cores from the same plot were mixed and placed in labelled plastic bags. Rocks and Large organic debris were removed using a 3.35-mm sieve. The samples were shipped with cold packs and processed for DNA extraction (FastDNA Kit for soil, MP Biomedicals) immediately. Extra soil samples were stored at -80 °C for references. Sample DNA was extracted from a 0.5-g sample from each plastic bag, and then quantified by a Nanodrop spectrophotometer 2000c (Thermo Scientific, Wilmington, Delaware). DNA samples were stored at -20 °C before sequencing.

Illumina sequencing

To investigate the entire soil microbial communities in soil with different treatments (rotation crops), Illumina Miseq was employed in this research. Samples associated with onion, corn and pumpkin were excluded due to lack of confidence (poor crop coverage). Among these samples, 37 were drawn from

Field A: 5 (3 at planting and 2 at harvest) drawn in 2014 for background references, and 32 (8 at planting and 24 at harvest) were from 2015. In total, 63 soil samples were drawn from Field B: 15 (3 at planting and 12 at harvest) drawn in 2014 for background references, and 48 (24 at planting and 24 at harvest) were from 2015. Soil DNA samples were validated by conducting conventional PCR with fungal universal primers ITS1/ITS4 (Manter and Vivanco 2007) and bacterial universal primers fd1/rp1(Akhtar et al. 2008) and determined the best work concentration of each in PCR. All the DNA samples were sent to the Research Technology Support Facility of Michigan State University (East Lansing, MI) for sequencing.

The amplicons were sequenced following Schmidt et al.'s (2013) and Kozich et al.'s (Kozich et al. 2013) protocols to analyze soil fungal and bacterial communities. From each soil DNA sample two NGS metagenomic amplicon libraries were generated. The V4 hypervariable region (515f/806r) of the bacterial 16S rRNA gene was amplified using complete, dual-indexed Illumina compatible adapters following a published protocol (Kozich et al. 2013). The second region targeted was the fungal internal transcribed spacer 1 (ITS1). For the ITS1 amplicons a two-step PCR design was used; the first step PCR targets the fungal ITS1 region with primers ITS1F12/ITS2 (Schmidt et al. 2013), with Fluidigm CS1 and CS2 oligomers added to their 5' ends. In the secondary PCR, dual-indexed Illumina compatible adapters are added using primers targeting the Fluidigm CS oligos at the ends of the primary PCR products. All PCR products were normalized using Invitrogen SequalPrep DNA Normalization plates, and products

recovered from the normalization plates were pooled. Separate pools were made for 16S-V4 and ITS1 amplicons. Each of the pools was given a final cleanup with AmpureXP magnetic beads and then quantified using Qubit dsDNA HS, Caliper LabChipGX HS DNA and Kapa Biosystems Illumina Library Quantification qPCR assays. The two pools were then combined in equimolar amounts for sequencing.

Sequencing was done in a 2 x 250bp paired end format (PE250) using a Illumina MiSeq v2 flow cell and 500 cycle reagent cartridge. Custom sequencing and index read primers were added. Base-calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.8.4.

Sequencing Outcome

The quality of sequencing results was high. The sequencing generated 13,950,218 raw clusters, and 11,679,139 of them passed the filter. Quality score Q-30 is a prediction of the probability of an error (0.001) in base calling (Illumina 2014). The overall percentage of bases with a quality score greater than Q30 (error rate less than 0.001) was averaged across the entire run, and it was 68.7%. The data analysis of sequencing results is undergoing. It is anticipated that Operational Taxonomic Units (OTUs) will be formed with the sequencing data, and OTUs will be used to compare the difference of the microbial community patterns (structure and composition) in the soil samples associated with different rotation crops.

BIOGRAPHY OF THE AUTHOR

Xuemei (Missi) Zhang was born in Neijiang, a small city of Sichuan Province in China, in 1991. She grew up with her brother in a happy family in Zizhong, Sichuan. When she was in China, she attended Southwest University in 2009 and graduated with her bachelor's degree in 2013. In 2013, she was accepted by the Plant, Soil and Environmental Sciences Department of The University of Maine. She joined Hao lab and started her master program in Spring 2014. During her master's study, she mainly worked on potato disease management and focused on potato pink rot control. She had several projects on the fungicide efficacy evaluation, fungicide resistance and crop rotation involved in pink rot control. In Fall 2016, prior to the completion of her master's degree, Xuemei decided to pursue her PhD degree in plant pathology, and she was accepted by the Department of Plant Pathology, Physiology and Weed Science of Virginia Polytechnic Institute and State University. She will start her PhD study right after she receives her master's degree. Currently, Xuemei is a candidate for the Master of Science degree in Plant, Soil, and Environmental Sciences of The University of Maine in December, 2016.