PHENOTYPIC CHARACTERISATION OF POTATO LATE BLIGHT PATHOGEN *PHYTOPHTHORA INFESTANS* IN BALTIC COUNTRIES

KARTULI-LEHEMÄDANIKU TEKITAJA *PHYTOPHTHORA INFESTANS* BALTIKUMI POPULATSIOONIDE FENOTÜÜBILINE ISELOOMUSTAMINE

ALICE AAV

A Thesis
submitted for the degree of Doctor of Philosophy
in Agriculture

Väitekiri
Filosoofiadoktori kraadi taotlemiseks
põllumajanduse erialal

Tartu 2016

Eesti Maaülikooli doktoritööd
Doctoral Theses of the Estonian University of Life Sciences

Eesti Maaülikool
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Estonian University of Life Sciences

According to the verdict No 6-14/4-8, the Doctoral Committee of the Agricultural Sciences of the Estonian University of Life Sciences has accepted this thesis for the defence of the degree of Doctor of Philosophy in Agriculture

Opponent: Associate prof. **Jadwiga Śliwka**
                 Plant Breeding and Acclimatization Institute -
                 National Research Institute;
                 Młóchow Research Centre, Poland

Supervisors: Associate prof. **Eve Runno-Paurson**
                 Estonian University of Life Sciences, Estonia
                 Prof. **Marika Mänd**
                 Estonian University of Life Sciences, Estonia

Reviewers: Dr. **Asko O. Hannukkala**
                 Natural Resources Institute Finland (Luke)
                 Dr. **Reet Karise**
                 Estonian University of Life Sciences

Defence of this thesis:
Estonian University of Life Sciences, room 2A1, Kreutzwaldi 5, Tartu on June 20, 2016, at 10.15.

The English language was edited by Dr. Ingrid H. Williams and the Estonian by Dr. Luule Metspalu.

Publication of this thesis is supported by the Estonian University of Life Sciences, Department of Plant Protection.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is a review of the following papers; the references to the papers are given in Roman numerals in the text. The papers are reproduced by kind permission of the following journals: European Journal of Plant Pathology (**I**), Agronomy Research (**II**), Zemdirbyste-Agriculture (**III**) and Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz - Journal of Plant Diseases and Protection (**IV**).

I Runno-Paurson, E., Remmel, T., Ojarand, A., **Aav, A.**, Mänd, M. 2010. The structure of *Phytophthora infestans* populations from organic and conventional crops. European Journal of Plant Pathology, 128 (3), 373–383.

II Runno-Paurson, E., Kiiker, R., **Aav, A.**, Hansen, M., Williams, I. H. 2016. Distribution of mating types, metalaxyl sensitivity and virulence races of *Phytophthora infestans* in Estonia. Agronomy Research, 14 (1), 220–227.

III **Aav, A.**, Skrabule, I., Bimšteine, G., Kaart, T., Williams, I. H., Runno-Paurson, E. 2015. The structure of mating type, metalaxyl resistance and virulence of *Phytophthora infestans* isolates collected form Latvia. Zemdirbyste-Agriculture, 102 (3), 335–342.

IV Runno-Paurson, E., Ronis, A., Hansen, M., **Aav, A.**, Williams, I. H. 2015. Lithuanian populations of *Phytophthora infestans* revealed high phenotypic diversity. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz - Journal of Plant Diseases and Protection, 122 (2), 57–65.

Table 1. Author's contribution to each article.

	Idea and design	Sampling	Laboratory work	Data analysis	Manuscript preparation
I	ERP	ERP, AO	ERP, AO	TR, ERP, MM	ERP, TR, **AA**
II	ERP	ERP, MH, RK	RK, MH, ERP	ERP, RK	ERP, RK, **AA**, MH, IHW
III	**AA**, ERP	**AA**, ERP, MH, IS, GB	**AA**, ERP, MH	TK, **AA**	**AA**, ERP, TK, IS, IHW, GB
IV	ERP	AR, **AA**, ERP	**AA**, MH, ERP	ERP	ERP, AR, MH, **AA**, IHW

AA – Alice Aav; AO – Ann Ojarand; AR – Antanas Ronis; ERP – Eve Runno-Paurson; GB – Gunita Bimšteine; IHW – Ingrid H. Williams; IS – Ilze Skrabule; MH – Merili Hansen; MM – Marika Mänd; RK – Riinu Kiiker; TK – Tanel Kaart; TR – Triinu Remmel; All – all authors of the paper

1. INTRODUCTION

Potato late-blight is one of the most destructive diseases of potato (*Solanum tuberosum* L.) worldwide; it is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary. The pathogen has the ability to cause considerable yield loss on potatoes and tomatoes during both the growing season and in storage. It has been studied for a long time by many scientists and the disease is known worldwide, because potato is such an important food crop worldwide. The biggest potato producing countries in the world are China, Russia and India according to FAOSTAT, 2014. Thousands of papers are published each year considering *P. infestans* (Fry *et al.*, 2015). Despite all this research, potato late-blight remains a huge problem in potato production and one of the worst crop diseases worldwide. Fungicides help to prevent and control the disease, and the number of applications has increased (Hannukkala *et al.*, 2007; Hannukkala 2012). However, frequent and regular application is needed (Cooke *et al.*, 2012) and due to their high price their usage is not always as strict as it should be to avoid the disease.

Potato late-blight is also a serious problem for potato growers in the Baltic countries. In Estonia, under favourable conditions, late blight can destroy the whole potato haulm with average losses reaching 20–25% and even more in fields not treated with fungicides (Runno-Paurson *et al.* 2010). In conventional potato production in Estonia, high yield with high quality cannot be achieved without fungicides (Koppel, Runno, 2006), so applications are made routinely; however, under favourable conditions for the disease, with heavy pressure from the pathogen, protection of large areas is complicated (Runno-Paurson *et al.* 2010). Furthermore, potato growers tend to choose West-European early varieties (Koppel, Runno, 2006), which have a high yield and good quality (Runno-Paurson *et al.*, 2013a), but have proven to be susceptible to the disease in Estonian conditions and therefore need intense chemical control with fungicides (Runno-Paurson *et al.*, 2009, 2013a). In Latvia, potatoes are an essential food, used for production of starch and chips, with those of lower quality being turned into animal feed (Cudere, 2008). Skrabule (2010) showed that in weather conditions favourable for the development of the disease, an organic potato production field, where no fungicides were used, was infected two weeks earlier than a conventional production field with applied fungicides. The average loss of yield in Latvian potato production

each year due to late-blight is 15–30% (I. Skrabule unpublished data). Potato growers in Lithuania suffer from the same problem, if the weather during the vegetation period is favourable for *P. infestans*, then the average loss of yield can go up to 50% (Ronis, Tamošiunas, 2005).

According to Estonian Statistical Database (2015), the total area under potato production has decreased 59% in the last ten years in Estonia, with large scale productions (more than 5 ha) now covering 57% of the land used for producing potatoes. In Latvia, 97% of the farms produce potatoes on less than 1ha, whereas in Lithuania 90% of farmers do so on less than 5ha. Most of the growers use no or a very low amount of fungicide and so the majority of potato fields are unprotected against late blight (Ronis, Tamošiunas, 2005; Cudere, 2008).

In Estonia, the advised rotation between potato fields is 3–4 years and on large scale productions, potatoes are planted not more often than every 3rd year (Runno-Paurson *et al.*, 2010; Runno-Paurson *et al.*, 2016). However in small scale production, potatoes are grown on the same field for years and the seed material is own multiplied from a mix of different cultivars (Runno-Paurson *et al.*, 2013b). In Lithuania, the rotation system in conventional farms is managed to 3–4 years between potato fields (personal communication with A. Ronis). However, the proportion of small scale producers is high (90%) in Lithuania (Ronis, Tamošiunas, 2005) and the rotation system is as weak sas in Estonian small scale productions (Runno-Paurson *et al.*, 2013b).

Resistance of different potato varieties against the disease is a crucial factor in choosing seed material, because this plays an important role in late blight development and thereby can reduce the potential loss of yield caused by *P. infestans*. Less damage was found in varieties with higher resistance in Denmark, Estonia, Poland, Lithuania and Latvia (Hansen *et al.*, 2005). Even existing higher resistance in different potato varieties does not eliminate the late blight problem, because growers use mostly susceptible or quite susceptible varieties for potato production, due to their earliness and high yield (A. Ronis unpublished data; Runno-Paurson *et al.*, 2013a). A recent study by Asakavičiūtė *et al.*, (2013) reported the cultivar 'Aista' to be the most resistant in Lithuania; however its other characteristics, such as lateness, high starch content (up to 21%) and taste do not favour it for cultivation (Asakavičiūtė *et al.*, 2009).

Treating potato plants with fungicides against late-blight as often as needed, is the only efficient way to control the disease and maintain high yields in conventional farming. However, there have been major changes in the populations of *P. Infestans*; the 'old' A1 mating type population, which was simple and reproduced only asexually, has been replaced with a 'new', more aggressive and diverse population, containing two mating types A1 and A2 (Fry *et al.*, 1993; Cooke *et al.*, 2011), which enables the pathogen to reproduce sexually. As a result of sexual reproduction, the pathogen can overwinter in the soil without the host plant – as oospores (Turkensteen, 2000). These changes in the pathogen populations have had a great impact on fungicide application strategies. Outbreaks of the disease in fields are occurring one month earlier and so the timing of the first fungicide treatment as well as the treatment intervals have to be adjusted according to the pathogen life cycle (Koppel, Runno, 2006; Cooke *et al.*, 2012).

P. infestans populations in Estonia have been studied since 1966 by researchers of the All Union Research Institute of Phytopathology who carried out the identification of virulence races (Koppel, 1996; Runno-Paurson, 2010). The A2 mating type was first mentioned in 1987 in Estonia (Vorobyeva *et al.*, 1991). The population studies of *P. infestans* in Estonia with phenotypic and genotypic characterisation restarted in the period 2001–2007. Research, considering the pathogen populations has continued ever since in Estonia.

In Latvia, data on *P. infestans* population characteristics is insufficient and out of date. During 1974–1990, identification of the pathogen races was made by the Institute of Phytopathology of Soviet Union (Bebre *et al.*, 2004). The A2 mating type was first mentioned in 1987 (Bebre *et al.*, 2004). There is information about oospore formation in Latvian potato fields, but no data about mating types (A1 and A2) (Bimšteine, 2008).

In Lithuania, some information about their populations of *P. infestans* comes from research carried out at the Lithuanian Institute of Agriculture, during 1990–1996, on metalaxyl (active agent in systemic fungicides) resistance (Valskytė, 2000). There is no information about A2 mating type registration or phenotypic genotypes.

Thus potato late blight has considerable importance in Estonian, Latvian and Lithuanian potato production, and with this little and out of date

data about the characteristics of the destructive pathogen in Latvia and Lithuania, this study was needed to try to fill the gap in the European population map for *P. infestans*. Due to the consequence of sexual reproduction, there is an ongoing change in genotypes and continuous diversification occurring in the Estonian *P. infestans* population, which should be studied continually (Runno-Paurson *et al.*, 2016). Understanding of the characteristics of *P. infestans* populations is essential for creation of an effective management system to control the disease (Cooke *et al.*, 2012).

2. REVIEW OF THE LITERATURE

2.1. Potato late blight and its causal pathogen
Phytophthora infestans

Phytophthora infestans (Mont.) de Bary is probably the most famous *Oomycetes*. It causes one of the most dreadful potato diseases – late blight (Figure 1), which is capable of infecting the whole plant (Fry, Smart, 1999). The symptoms at the beginning of the disease are light to dark green, irregular water-soaked spots on the potato leaves, which usually start to develop near the leaf tips or edges. These spots expand rapidly and turn to dark brown or black lesions (Kirk *et al.*, 2004). In moist conditions, greyish-white mycelia appear on the lower surface of the leaf (Figure 1). Tubers can be affected as well. When they come into contact with spores of *P. infestans* washed down from infected leaves by rain or heavy dew, then infection takes place (Lacey, 1967). The symptoms are grey or dark spots on the tuber surface and reddish-brown soft bad-smelling tissue underneath the skin (Figure 2). If the infected tubers are stored in cool, well-ventilated conditions, then little or no infection from tuber to tuber occurs (Kirk *et al.*, 2004).

Figure 1. One symptom of *P. infestans* infection – greyish-white mycelia on the lower side of the potato leaf (Photo: Alice Aav, 2012).

Figure 2. *P. infestans* infection in potato tuber (Photo: Eve Runno-Paurson, 2012).

As the pathogen is able to reproduce very quickly and its genetic flexibility is high, it can cause serious damage to potato production (Hannukkala, 2012). The economic losses due to late blight are estimated at over €1,000 million a year in Europe, including the costs of control and damage (Haverkort *et al.*, 2008). In developing countries, where farmers cannot afford to buy fungicides, the disease can completely destroy the potato crop (Rubio-Covarrubias *et al.*, 2005). The explosive potential of the pathogen is derived from its rapid reproduction – its life cycle can be completed within 4 days if weather conditions are suitable, while at the same time the sporangia can spread kilometres between the potato fields (Fry, 2007) and this explains why whole fields can be destroyed within a few days (Fry, 2008).

2.2. *Phytophthora infestans* origin and migration to Europe

There are two major theories about the origin of *P. infestans*; one that it came from the Andes, South-America (Gomez-Alpizar *et al.*, 2007), the other that it came from Toluca Valley, central highlands of Mexico (Grünwald, Flier, 2005). A recent study by Goss *et al.*, (2014) which compared the two possible origins with specific methods, strongly supports Mexico as the origin.

Historically, two major migrations of *P. infestans* from Mexico to Europe have taken place. The first reports in Europe about late-blight came

from Belgium, Ireland and Germany in 1845 and it is suspected that the pathogen was brought to Europe with infected seed tubers straight from Mexico or through the United States (Fry *et al.*, 1993), so this was the first migration. At that time the pathogen population worldwide contained only A1 mating types, with the exception of central Mexico (Fry *et al.*, 2009). Soon after the first migration, the Irish potato famine happened, which resulted in replacement or death of ⅓ of Ireland's population (Drenth *et al.*, 1994). The efforts in the following years, after the *P. infestans* epidemics in Europe, to supress and control the disease succeeded and by the late 1970s the disease was not as important as it had been during the previous century (Fry, Smart, 1999). Gisi and Cohen (1996) reported that the A1 mating type dominated in most European populations until the early 1980s.

The second migration occurred in the late 1970s with which the A2 mating type, previously found only in Mexico, was brought to Europe together with genetically diverse and aggressive strains of the pathogen (Fry *et al.*, 1993). The first report, confirming the presence of the A2 mating type, came from Switzerland in 1981 (Spielman *et al.*, 1991), and since then occurrence of the A2 mating type has been confirmed from almost every European country (Gisi, Cohen, 1996). The "new" strains of *P. infestans* replaced the "old" strains very fast and earlier outbreaks of the disease became possible (Fry *et al.*, 1993; Drenth *et al.*, 1994). The old clonal lineage is now found only rarely in Europe (Cooke *et al.*, 2011).

2.3. Ecology and epidemiology of *Phytophthora infestans*

2.3.1. Life cycle of *Phytophthora infestans*

P. infestans is a heterotallic oomycete with two mating types A1 and A2 and the pathogen can reproduce both asexually and sexually (Gallegly, 1968). Furthermore, both mating types, irrespective of the opposite mating type, can reproduce asexually (Yuen, Andersson, 2013). Asexual reproduction allows the pathogen to distribute easily and grow the population very fast (Fry, 2008; Turano, 2015). Coexistence of the mating types allows the pathogen to reproduce sexually and, as a result, to form oospores (Turkensteen, 2000) which are able to survive in the soil for up to ten years (Drenth *et al.*, 1995). Sexual recombination gives the pathogen an evolutionary advantage through increasing genetic diversity and oospores

provide a source of primary inoculum in the soil, as well as enabling the pathogen to survive in unfavourable conditions (Turano, 2015). Furthermore, sexual reproduction also changes the way of approaching disease management (Barton, Charlesworth, 1998; Cooke *et al.*, 2012; Yuen, Andersson, 2013). However, *P. infestans* is acclimatizing well to different climatic conditions, ensuring its survival (Yuen, Andersson, 2013).

The asexual life cycle (Figure 3). Moisture, especially the free moisture on leaf surfaces, is very important for *P. infestans*, because zoospores swim in the water on the leaf surface before invading the plant tissue. At the same time, the duration (at least 12h) of leaf wetness is important as well, because this increases sporulation (Fry, 2007). The spores can go through direct germination (germination tube is formed straight away – it can take place in dry or wet conditions) or indirect germination (sporangiums are formed, which release zoospores – it can take place in wet conditions) (Turkensteen, 2005). Temperature is the second important factor for the
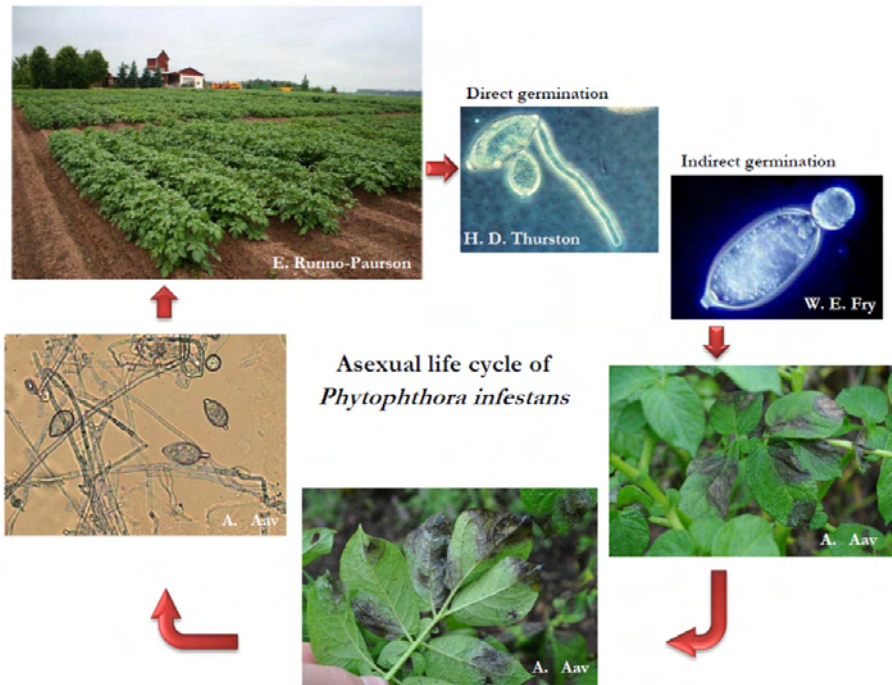

Figure 3. Asexual life cycle of *P. infestans* (idea and design of the figure originates from Turano, 2015).

development of the pathogen. The optimum temperature range for *P. infestans* is 16–20 °C, the highest amount of sporangia are produced at 24 °C and the optimum temperature for sporangia to release zoospores is at 15 °C (Sujkowski, 1987). Temperatures lower than 15 °C or higher than 25 °C inhibit the growth and development of the pathogen (Fry, 2007).

Microscopically, there are no visible late blight symptoms right after the infection, but after at least 2 days, circular to irregular-shaped water-soaked spots appear; their development starts from the leaf tips or edges and they grow fast into large dark brown lesions (Kirk *et al.*, 2004). Then after 1–2 days, under favourable conditions (cool and moist), sporangiophores with sporangia are produced (Fry, 2008). The main distribution of the disease in the field is through asexual spores, which are blown for kilometres by wind to neighbouring fields. Spread of the disease depends on weather conditions as well as on the potato growing period at the time of infection (Razukas *et al.*, 2008).

There are populations in the world, where the pathogen reproduces mainly asexually, for example in North- and South America, Asia and Africa (Yuen, Andersson, 2013) and the population structure in European countries – Great Britain, France, Switzerland, Netherlands, Belgium and Denmark is clonal with only a few genotypes that dominate the population and sexual reproduction is rare (Montarry *et al.*, 2010; Gisi *et al.*, 2011; Cooke *et al.*, 2012; Li *et al.*, 2012).

The sexual life cycle (Figure 4). For sexual reproduction the pathogen needs both mating types, A1 and A2, to be present, after which male antheridia and female oogonia are produced (Fry, 2008). After fertilisation, thick-walled oospores are formed (Turkensteen *et al.*, 2000). In favourable conditions, oospores germinate and new sporangia are released (Turkensteen *et al.*, 2000).

Sexual reproduction did not occur in European populations of the pathogen before the 1970s, as only one mating type A1 was present (Spielman *et al.*, 1991; Drenth *et al.*, 1994). However nowadays, there is evidence from northern Europe, that regular sexual reproduction is taking place (Yuen, Andersson, 2013) and the characteristics in northeastern European populations indicate sexual recombination as well (Lehtinen *et al.*, 2007, 2008; Runno-Paurson *et al.*, 2009, 2010, 2011, 2012, 2013b, 2014; Hannukkala, 2012; Statsyuk *et al.*, 2013).
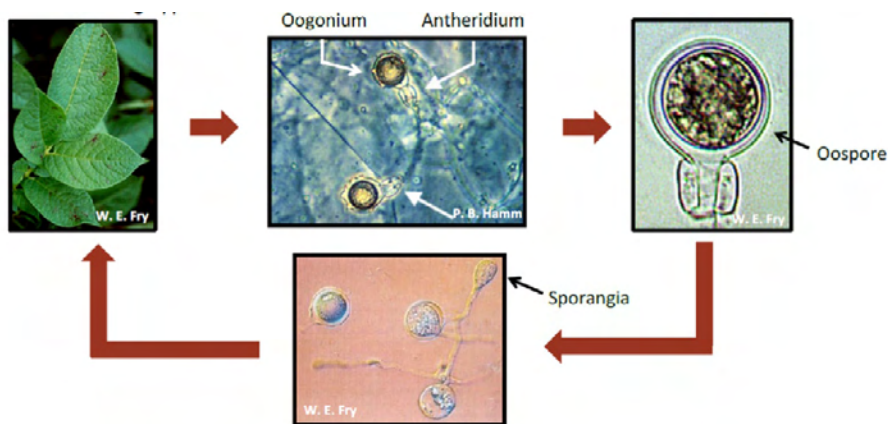
Figure 4. Sexual life cycle of *P. infestans* (Turano, 2015).

2.3.2. *P. infestans* overwintering

The asexual population of *P. infestans* overwinters as mycelia in stored infected tubers; the hosts can be potatoes (in cooler climates), tomatoes as well as other tuberous *Solanum* species (in warmer climates) (Fry, *et al.*, 1993). It can also stay alive during winter in any tubers left in the soil during harvest. These can act as volunteer plants the next season (Zwankhuizen, Zadoks, 2002; Lehtinen, Hannukkala, 2004). The survival of the asexual *P. infestans* population relies completely on the viability of the tuber in storage, soil or waste pile (Hannukkala, 2012). Montarry *et al.*, (2007) points out that intermediately aggressive isolates are more likely to stay alive, because they do not kill all infected tubers before sprouting, as the aggressive strains do. If the temperature is low enough to freeze the soil, then the survival of the asexual stage of *P. infestans* is limited (Lehtinen, Hannukkala, 2004). The pathogen remains alive over the winter in the tubers in countries where the soil layer containing the volunteer tubers remain unfrozen. For example, it is thought that in French populations, *P. infestans* mainly overwinters as mycelia in potato tubers in the soil or in refuse piles near potato fields (Montarry *et al.*, 2007).

Slow disease progress and long epidemic duration creates good conditions for oospore formation, whereas the fast epidemic process results in

rapid decay of the plant with production of few or no oospores (Romero-Montes, 2008). These thick-walled spores are formed in potato tissues and fall to the ground together with plant residues; once in the soil, no host plant is needed for survival (Drenth *et al.*, 1995; Mayton *et al.*, 2000; Hannukkala, 2012) turning *P. infestans* into a soil borne pathogen. Furthermore, oospores can tolerate very low temperatures (Drenth *et al.*, 1995). Romero-Montes *et al.*, (2008) reported from different studies that the quantity of oospores formed depends on the number of lesions per leaf, on the resistance level of a particular cultivar, and on the temperature. In the next vegetation period the germinating oospores can cause an infection in newly planted potatoes and this may result in early epidemics in potato fields (Lehtinen, Hannukkala, 2004; Bødker *et al.*, 2006; Brylińska *et al.*, 2016). Oospores have an important role in pathogen survival, especially in the Nordic climate, where the temperatures on the winter time drop below 0 °C and the soil freezes (Hannukkala, 2012), because the asexual pathogen population could not survive in such conditions in frozen tubers (Lehtinen, Hannukkala, 2004). Oospores remain viable for 3–4 years according to studies in Nordic and Dutch environmental conditions (Drenth *et al.*, 1995; Turkensteen *et al.*, 2000; Cooke *et al.*, 2011).

2.4. *P. infestans* population studies in Europe

2.4.1. Markers used for characterising the diversity of *P. infestans* populations

To characterise pathogen populations, certain markers for determing traits are needed (Fry *et al.*, 2009). Cooke and Lees (2004) reviewed the different markers used to investigate populations of *P. infestans* and reported that the most studied phenotypic characteristics of the pathogen are: mating type (Gallegly, Galindo, 1957), virulence (Malcolmson, Black, 1966) and resistance to fungicide (Dowley, O'Sullivan, 1981). These phenotypic markers have been used in most of the *P. infestans* population studies in Europe and are used in this thesis.

The first phenotypic marker, mating type, is determined by a robust analysis, where the isolate under investigation is paired with both of the known mating types A1 and A2; if oospores are formed in contact with the A1, but not with A2 mating type, the analysed isolate has A2 mat-

ing type (Fry *et al.*, 2009). However, there is a slight weakness in this method, due to the fact that other factors, such as age of cultures, various substrates (for example oats, lima beans etc.) or physical wounding can sometimes induce the formation of oospores (Fry *et al.*, 2009). Still, information of the mating type ratios is important for understanding population diversity and disease aetiology (Cooke, Lees, 2004).

The second phenotypic marker, often used to give insight to a pathogen population structure, is response to metalaxyl (a phenylamide fungicide with systemic function) (Fry *et al.*, 2009). This fungicide had a major effect in controlling potato late blight, but the first metalaxyl resistant strains were already present in the late 1970's as described by Dowley and O'Sullivan (1981) (Fry *et al.*, 2009). If the frequency of metalaxy-resistant isolates in the population is high, the use of metalaxyl-based products may fail.

Virulence to a specific resistance-gene is the third phenotypic marker often used to analyse the population; furthermore to get the answers, the specific R-genes, derived from wild *Solanum* species (Malcholmson, Black, 1966), can be useful in resistance breeding (Fry *et al.*, 2009). An isolates 'race', or virulence pathotype, is verified by inoculating 11 'differential' genotypes, each containing a different R-gene and by assessing the infection rate after seven days (Cooke, Lees, 2004). This analysis is useful when comparing different populations of the pathogen (Fry *et al.*, 2009). The concept of "virulence" testing is somewhat out of date but still applicable for population studies. The recent genetic studies have revealed that many former "major R-genes" actually are not single genes and the latest approach to resistance names the resistance generating factors "effectors", instead of virulence or resistance genes (Cooke *et al.*, 2012). Furthermore, additional resistance genes have been identified and cloned from wild *Solanum* species, such as *S. bulbocastanum*, *S. stoloniferum*, *S. chacoense*, *S. venturii* and *S. mochiquense* (Vossen *et al.*, 2011; Sun *et al.*, 2016).

Phenotypic characteristics are important for describing population structure, but these analyses do not give all the information needed about the *P. infestans* pathogen (Cooke, Lees, 2004). Therefore, genotypic analyses (in alphabetical order), such as allozymes, amplified fragment length polymorphisms (RFLPs), mitochondrial haplotypes, restriction fragment length polymorphisms (RFLPs) and simple sequence repeats

(SSRs or microsatellites) have been developed and are widely used (Fry *et al.*, 2009). Since the research carried out and presented in the current thesis did not involve genotypic analyses, these methods are mentioned but not further discussed.

2.4.2. Short overwiew of the populations of *P. infestans* in Europe

The *P. infestans* populations in Western Europe are very different from those in the Nordic countries. The pathogen populations in Great Britain, France, Switzerland, Netherlands, Belgium and Denmark are clonal with few genotypes and infrequent sexual reproduction (Gisi *et al.*, 2011; Cooke *et al.*, 2012; Li *et al.*, 2012; Montes *et al.*, 2016). For example, in France, although both mating types are present, there is no evidence that sexual reproduction takes place (Montarry *et al.*, 2010). Changes in the genetic structure can still occur, as in Great Britain, new clonal lineages have appeared, replaced the old predominant lineages, and come to dominate the *P. infestans* population (Cooke *et al.*, 2012). In Western Europe, the pathogen survives over winter mainly as mycelia in infected tubers (Cooke *et al.*, 2012; Li *et al.*, 2012). There have been years when the metalaxyl-resistant strains dominate the population in Ireland, France and United Kingdom (Duvauchelle *et al.*, 2009; Cooke *et al.*, 2012; Gisi *et al.*, 2011). The most frequent virulence race is the same in Europe and in the Nordic countries – 1.3.4.7.10.11 (Hermansen *et al.*, 2000; Lehtinen *et al.*, 2008; Hannukkala, 2012; Runno-Paurson *et al.*, 2014).

The pathogen populations in northern and northeastern Europe, are diverse, since both mating types are present in potato fields, and the ratios between A1 and A2 favour sexual reproduction; therefore the phenotypic variability is very high in the pathogen populations of these regions (Lehtinen *et al.*, 2007, 2008; Runno-Paurson *et al.*, 2009, 2010, 2011, 2012, 2013b, 2014; Hannukkala, 2012; Statsyuk *et al.*, 2013, Brylinska *et al.*, 2016). Yuen and Andersson (2013) have found clear evidence, which indicates regular sexual reproduction in the Nordic countries. The pathogen can survive over winter as a mycelium in the tuber (limited with cold winters) and as oospores in the soil (Hannukkala, 2012). Metalaxyl-sensitive strains of *P. infestans* dominate the populations in the Nordic countries (Lehtinen *et al.*, 2008).

2.5. Methods for controlling potato late-blight disease

Control of potato late blight disease is possible but needs regular and frequent use of fungicides (Cooke *et al.*, 2012). Early outbreaks of the disease, needing more applications of fungicide, have been reported from Nordic regions (Lehtinen, Hannukkala, 2004). Many potato growers treat their potato plants routinely from when plants have grown to touch neighbouring plants in a row until harvest (Cooke, Lees, 2004). Large scale commercial growers in Estonia apply fungicides approximately 6 to 7 times per season (Runno-Paurson *et al.*, 2009). In Latvia, potato fields up to 0.5 ha are on average treated only once, whereas growers with larger areas of potato treat 3 to 5 times (Cudere, 2008). In Lithuania, growers with more than 5 ha of potato crop treat with fungicides on average 4 to 6 times per season (Valskytė, 2000; Ronis, Tamošiunas, 2005).

In conventional potato production fungicide treatments are permitted and are made routinely, but this certainly has negative effects on the environment (Yao *et al.*, 2016). However, in organic management practices, the most important method for controlling the disease is prevention – proper crop rotation, certified disease-free seed material, choosing resistant varieties, weed management, and destruction of possible inoculums (refuse piles, voulenteer plants etc.). Copper based fungicides are allowed in organic management practices in Europe, including in Lithuania, but are prohibited in Estonia.

To lessen the negative impact on the environment, biological control methods have been studied. A variety of commercial products, based mainly on the genus *Trichoderma* have been developed and can be used against crop pathogens, including *P. infestans* (Elad, Kapat, 1999; Yao *et al.*, 2016).

More resistant cultivars can be grown to protect the canopy from infection, but potato growers tend to choose susceptible or quite susceptible potato varieties due to their earliness and quality. Potato breeders have developed varieties relatively resistant to late-blight, but changes in *P. infestans* race structure weaken the created resistance (Asakavičiūtė *et al.*, 2013). The level of resistance is also dependent on variation in soil and climate (Tatarowska *et al.*, 2012) which can influence the development of the disease. Runno-Paurson *et al.*, (2013a) survey of resistance evaluation showed, that popular western European potato varieties are

not resistant enough to be grown without fungicide application under North-East European conditions. Furthermore, Razukas *et al.* (2008) reported that none of the potato cultivars grown in Europe have adequate late blight resistance. Still, some cultivars are more resistant to the disease than others, for example, in Lithuania, cultivar 'Aista' is partially resistant to late-blight, but is a late variety with a high starch content (up to 21%) and therefore is not chosen for production by local farmers (Asakavičiūtė *et al.*, 2009; Asakavičiūtė *et al.*, 2013). Breeding resistant varieties is important, but quite challenging, because *P. infestans* has mutable features and can break through any resistance in the long term (Razukas *et al.*, 2008). Asakavičiūtė *et al.*, (2013) reported that in the long-term, breeding resistant varieties using the "major resistance genes", may fail. Yuen and Andersson (2013) conclude as well that using specific resistance genes (R-genes), as a preventative method against potato late-blight, can be useless in the future due to the pathogen's ability to break through the resistance created with these genes. *P. infestans* is a highly changeable pathogen, which easily adjusts to the factors limiting its development, such as resistance created with R-genes (Tatarowska *et al.*, 2012).

3. AIMS OF THE STUDY

The novelty of this study was to fill the information gaps about the phenotypic characteristics in the populations of *P. infestans* in Latvia and Lithuania as one part of Eastern Europe. Due to sexual reproduction, there is an ongoing change in genotypes and continuous diversification occurring in Estonian *P. infestans* population, which should be studied continually (Runno-Paurson *et al.*, 2016). Therefore, the main aims of the this study were to determine the general characteristics of the populations of *P. infestans* in the Baltic countries, in terms of mating type, reaction to metalaxyl and pathotypic diversity (**I**, **II**, **III**, **IV**).

More specifically we were interested to find out:

1) What is the phenotypic variation within the Estonian (**I**, **II**), Latvian (**III**) and Lithuanian (**IV**) populations of *P. infestans* indicated by mating type, metalaxyl resistance and virulence and how does it vary between Baltic countries?

2) Does the mating type ratio in Latvia and Lithuania suggest occurrence of sexual reproduction (**III**, **IV**)?

3) What are the impacts of time (**I**, **II**, **III**, **IV**), site (**II**, **IV**) and the influence of agricultural management practices (**II**, **III**, **IV**) on studied population characteristics of *P. infestans* in the Baltic countries?

The hypothesis of the study:

• The populations of *P. infestans* in Estonia, Latvia and Lithuania are diverse and highly complex.

• Mating type ratio in Estonian, Latvian and Lithuanian populations of *P. infestans*, indicates possible sexual reproduction of the pathogen on the studied fields.

• The populations of *P. infestans* are similar in the Baltic countries.

• Differences derived from time, site and agricultural management practices are present in the populations of *P. infestans* in the Baltic countries.

4. MATERIALS AND METHODS

4.1. Collection and isolation of *P. infestans* isolates

4.1.1. Study sites and isolates sampling

In 2004–2005, 196 *P. infestans* isolates were collected from twelve sites (potato fields) in northern Estonia (**I**). There were 4 organic, 4 small scale conventional (SSC) and 4 large scale conventional (LSC) fields (Table 2).

In 2013, 110 samples of *P. infestans* were collected from nine sites from five counties in Estonia, utilising four different agricultural management practices – LSC, SSC, organic and potato trial fields (Table 1, **II**). During 2010–2012, 181 isolates of *P. infestans* were collected from 23 sites from 13 locations in Latvia (Figure 1, Table, **III**). The samples were collected from four different agricultural management practices – LSC, SSC, organic and potato trial fields. During 2010–2012, 93 *P. infestans* samples were collected from 22 sites from four regions in Lithuania (Figure 1, Table 1, **IV**). The samples were collected from three types of agricultural management practices – LSC, SSC and experimental field plots.

Table 2. Sampling of *P. infestans* isolates collected from Estonia during 2004–2005 (**I**).

Site	Agricultural management practice	Tested for		
		Mating type (n)	Metalaxyl (n)	Virulence (n)
Ingliste 2004	LSC	22	20	22
Ingliste 2005	LSC	23	23	25
Kõue 2004 1	SSC	17	4	15
Kõue 2004 2	SSC	18	3	23
Kõue 2005 1	SSC	16	9	16
Kõue 2005 2	SSC	10	3	14
Laheotsa 2004	LSC	17	17	17
Laheotsa 2005	LSC	10	10	10
Turba 2004 1	Organic	3	5	9
Turba 2004 2	Organic	9	5	12
Turba 2005 1	Organic	13	9	15
Turba 2005 2	Organic	17	2	18
TOTAL		175	110	196

On the LSC farms, certified seed potatoes of high quality were used (**I**, **II**, **III**, **IV**). Fungicides were applied in Estonia (2004–2005) 6–7 times (**I**); in 2013 6–8 times, whereas Ridomil Gold MZ 68 WG (metalaxyl based fungicide) was used at least twice at the beginning of the outbreak of the disease (**II**); in Latvia 3–5 times (**III**) and in Lithuania 4–7 times (**IV**) per growing season. Also the crop rotation system was well organized and varied between 0–2 years (**II**) or 3–4 years (**I**, **III**, **IV**). On the SSC farms, seed potatoes of uncertain quality were used (**I**, **II**, **III**, **IV**) and crop rotation was not practiced (**I**) or varied between 1–2 years (**II**). Fungicides were not used routinely (**II**, **III**, **IV**) or used only once per growing season (**I**). On the organic field and field trials the rotation system was 3–4 years between potatoes.

All the isolates were collected from infected potato leaves, each with a single lesion and the plants, used for collection, were located at a random distance from field edges. Potato leaves were chosen randomly from the plant and leaves with more than one lesion were not taken. Collection of the isolates was carried out at the beginning of the outbreak of the disease (**I**, **II**, **III**, **IV**) with the exception of the SSC fields in Lithuania (**IV**) where technical problems prevented collection of leaves at the beginning of the outbreak of the disease, until the end of the growing season (up to 97% of the foliage destroyed), with the exception of the LSC farms in Estonia (**I**), where the leaves were collected only at the beginning of the outbreak. In the early stages of the disease, 10–25% of the leaf area of the infected potato plants and less than 10% of the plants in the field were infected with late blight; in the later stages the numbers were 20–40% and more than 50%, respectively.

4.1.2. Culture of isolates

To culture the pathogen, tubers of the susceptible cultivar 'Berber' (**I**, **II**, **III**, **IV**) or 'Bintje' (**I**), without known resistance genes (R-genes) were used. After sterilising the tubers in ethanol and flame, they were cut to slices. Infected leaf tissues were placed between the tuber slices, put into sterile Petri dishes and incubated in a growth chamber for 6–7 days at 16 °C, until the mycelia had grown through the slice. A small amount of mycelium was then transferred with a sterile needle to rye B agar (Caten and Jinks, 1968). The pure cultures were transplanted to rye B agar every 2 months. All phenotypic tests were carried out in September-November of the year of isolation.

4.2. Phenotypic analyses

4.2.1. Mating type determination

Mating type determination was carried out by growing each sample isolate together with the appropriate tester strain (A1 and A2) in a Petri dish on rye B agar (**I**, **II**, **III**, **IV**). Tester isolates, 90209 (A1) and 88055 (A2), were sent from the Natural Resources Institute, Finland, Luke by Asko O. Hannukkala (**I**, **II**, **III**, **IV**). After 10–18 days in the growth chamber at 16 °C, the area between tester strain and the isolate under examination was assessed for oospore formation. Determination was carried out under a microscope. Isolates forming oospores on plates with the A1 mating type but not the A2 were recorded as A2; isolates that formed oospores with the A2 mating type but not with A1 were recorded as A1; isolates that formed oospores with both A1 and A2 were recorded as self-fertile.

4.2.2. Resistance to metalaxyl

Resistance to metalaxyl was tested using a modification of the floating-leaflet method (Hermansen *et al.*, 2000) (**I**, **II**, **III**, **IV**). Leaves from approximately five-six week old plants of the susceptible cultivar 'Berber' were taken and 14–15 mm diameter disks (Figure 5) were cut with a cork borer.

Figure 5. Experiment for metalaxyl-resistance determination (Photo: Alice Aav, 2012).

Petri plates (50mm) were half-filled (7ml) with distilled water or water and metalaxyl solution with concentrations 0.0, 10.0 and 100.0 mg l^{-1} (prepared from technical grade metalaxyl-M, Syngenta experimental compound, CGA 329351A) and six leaflets were floated on the liquid in each plate. The discs were infected with a suspension of sporangia (20 µl drop) prepared from distilled water and the pathogen's sporangia grown in pure cultures on rye B agar. Petri dishes were placed in a growth chamber and maintained at 16 °C and 90% relative humidity. After seven days the isolates were assessed by the same parameters as described in Runno-Paurson *et al.* (2009) and recorded as resistant (leaflets sporulated in 100 mg l^{-1} metalaxyl), intermediate (sporulated in 10 mg l^{-1}, but not on 100 mg l^{-1} metalaxyl) or sensitive (sporulated only in distilled water/0 mg l^{-1} metalaxyl).

4.2.3. Virulence tests

The specific virulence was determined using Black's differential set of potato genotypes containing resistance genes R1–R11 (Malcolmson, Black, 1966) provided by the Scottish Agricultural Science Agency, United Kingdom (**I**, **II**, **III**, **IV**). Due to technical and logistic reasons, in 2010 only some of the isolates were tested for pathotypes (**III**) or were not tested at all (**IV**). The virulence testing was done using detached leaflets from the differentials (varieties, each containing one known resistance gene from R1–R11) grown from meristem plants in the greenhouse. The meristem plants were provided by the Plant Biotechnological Research Centre EVIKA (Saku, Estonia). Leaves, cut from 6–8 week old potato plants, were placed lower side up on moistened (distilled water) filter paper in plastic trays (Figure 6) and inoculated with a suspension of *P. infestans* sporangia (20 µl drop, 1.0–4.0 × 10^4 sporangia ml^{-1}). Polyethylene was used to cover the plastic trays to maintain high relative humidity after the inoculation. The plastic trays were kept at 16 °C and 16h light period.

After seven days the leaves were evaluated for virulence: 0 – no symptoms; 1 – small necrotic spots; 2 – <10% necrotic spot on the leaf; 3 – 10–50% of the leaf covered with mycelia; 4 – 50–75% of the leaf covered with mycelia, 5 – >75% of the leaf covered with mycelia. The trial was carried out in four replications.

Figure 6. Experiment for virulence testing (Photo: Eve Runno-Paurson, 2012).

4.3. Data analysis

Statistical analyses were performed with the SAS version 9.4 (**III**) and SAS/STAT version 9.1 (**I**, **II**, **IV**, SAS Institute Inc., Cary, NC, USA). Procedure GLIMMIX was used to test for effects of year, field type, and their interaction on metalaxyl resistance and mating type; multinomial models considering also random effect of site nested to field type were applied (**III**). To find the associations of metalaxyl resistance and mating type with sampling year by field types and with field types by sampling year, and to study the relationship between metalaxyl resistance and mating type, the Fisher exact test was applied (**III**). Logistic analysis (procedure GENMOD) with a multinomial response variable (A1, A2, or both) was used to test for differences in the distribution of the two mating types between years, study sites, regions and field types (**I**, **II**, **IV**). Analogous logistic procedures were used to test for differences in the resistance to metalaxyl between years, sites, regions, field types and between different mating types (**I**, **II**, **IV**). Average susceptibility of potato plants grown in different cropping systems to late blight was tested using a logistic model with an ordinal multinomial responses variable (**I**).

The race diversity was calculated with the normalized Shannon diversity index (**II**, **III**, **IV**). The differences in the values of the Shannon index between sites were analysed with one-way ANOVA and Tukey HSD test (**II**). Logistic model (SAS 9.4 procedure GLIMMIX) was used to perform pairwise comparison of virulence frequencies against different potato R-genes, at the same time considering effects of year, field type, their inter-actions with R-genes and random effect of site nested to field type and followed by the Tukey test for multiple comparison of least square means (**I**). The dependence of specific virulence on year, sites, field types and R-genes was analysed with type III ANOVA and Tukey HSD post-hoc tests (**II**, **IV**). "Site" was treated as a categorical variable in all analyses with Estonian isolates in 2013 (**II**). Fisher exact test was used to test the associations between virulence to single potato R-gene and metalaxyl resistance, mating type, year and field type (**III**). Each series of p-values concerning 11 potato R-genes was corrected for multiple testing using the Holm method (**III**). The dependence of race complexity on isolation time and the differences in the Shannon index values between years, sites and regions were analysed with one-way ANOVA and Tukey HSD test (**I**, **IV**). χ^2-goodness-of-fit test was applied to test the difference of A1 and A2 mating types from 1:1 ratio (**IV**).

Hierarchical cluster analysis and principal component analysis were per-formed to discover common patterns in virulence against R-genes (**III**). Principal component analysis was used also to study the relationships of sampling year, field type, mating type and metalaxyl resistance with common virulence patterns.

Statistical software *R 3.1.1* was used to perform Fisher exact tests and all multivariate analyses, for principal component analysis package *ade4* was applied (**III**). Test results with $p < 0.05$ were considered statistically significant (**I**, **II**, **III**, **IV**).

5. RESULTS

5.1. Mating type

The overall frequencies of mating types are presented in Table 3. Both mating types (A1 and A2) were found in Estonia in 2004–2005, from 11 of 12 fields (**I**) and in 2013 from all studied fields (**II**). Both *P. infestans* mating types were present also in Latvia (**III**) and Lithuania in all studied years, whereas A1 mating type was found from 16 and A2 mating type from 14 of 20 studied fields in Lithuania (**IV**).

The proportion of A2 mating types between two sampling years in Estonia differed significantly (Chi-square = 11.87, *df* = 1, *p* = 0.0006), being lower in 2004 (28%) and higher in 2005 (54%) (**I**). No statistically important difference in the distribution of mating types between years was found (*p* = 0.55, **III**) on the isolates collected from Latvia (2010–2012) and the interaction effect of year and field type was also statistically non-significant (*p* = 0.06, **III**). The percentages of A2 mating type frequency in studied years in Lithuania varied between 41–50%, still the difference between years was statistically non-significant (**IV**) in contrast to that in Estonia in 2005–2005 (**I**). Furthermore, there was no statistically significant difference in any of the studied years in Lithuania (2010 – Chi-square = 0.09, *p* = 0.76; 2011 – Chi-square = 0.45, *p* = 0.051; 2012 – Chi-square = 0.30, *p* = 0.58; **IV**).

The proportion of A1 and A2 between sampling sites differed significantly in Estonia in 2013 (Chi-square = 25.44, *df* = 8, *p* = 0.001), furthermore

Table 3. The frequencies of mating types in *P. infestans* populations (**I**, **II**, **III**, **IV**).

Country	Isolates tested	Mating type frequencies (%)		
		A1	A2	Self-fertile
Estonia (**I**)	175	57	41	2
Estonia (**II**)	110	71	29	0
Latvia (**III**)	181	53	43	4
Lithuania (**IV**)	93	50	45	5

the average incidence of A2 mating type varied between potato fields from 7 to 78% (Table 2, **II**). Significant differences in the proportion of A1 and A2 between sampling sites were also found in Lithuania (Chi-square = 84.49, *df* = 44, *p* = 0.0002, **IV**), whereas association between mating type proportions and different regions was also significant (Chi-square = 14.79, *df* = 6, *p* = 0.022), indicating the higher proportion of A2 mating type in the northern (16 out of 21) and eastern regions (8 out of 19) (**IV**).

The distribution of mating types between agricultural management practices was statistically non-significant in Latvia (*p* = 0.40) (Figure 2, **III**). However, significant association was found between mating type and agricultural management practices (*p* = 0.004) in 2010, when breeding and organic fields were not tested, and left out of the analysis. A2 mating type was more prevalent in large scale conventional fields (56.7% of findings), whereas the A1 mating type was present in all types of fields with almost the same frequency. This association between mating type and agricultural management practices in 2011 and 2012 persisted in general with some changes. A1 was found in 40.6% of large scale fields and in 9.4% of organic fields in 2011, but in 2012, the opposite was found, with A1 in 31.5% of organic fields and 15.8% of large scale conventional fields. The A2 mating type was most frequently found from large scale conventional fields in both years (2011 – 45.2%, 2012 – 66.7% of the cases). A2 mating type findings from breeding fields were quite different in numbers as well, 29.0% in 2011 but only 5.6% in 2012. Self-fertile mating type was present only in small and large scale conventional fields. (**III**). Differences between agricultural management practices in Lithuania were statistically non-significant (Chi-square = 3.11, *df* = 2, *p* = 0.54, **IV**).

5.2. Metalaxyl resistance

The overall frequencies of matalaxyl-resistance are presented in Table 4. Isolates with resistant, intermediate and sensitive responses to metalaxyl were present in all studied countries.

Response to metalaxyl between studied years was statistically non-significant in the Estonian population, in 2004–2005 (Chi-square = 0.98, *df* = 1, *p* = 0.42; **I**). The proportions in Latvia varied with year, but

Table 4. The frequencies of metalaxyl-resistance in *P. infestans* populations (**I, II, III, IV**).

Country	Isolates tested	Frequencies of metalaxyl-resistance (%)		
		Resistant	Intermediate	Sensitive
Estonia (**I**)	110	49	34	17
Estonia (**II**)	110	16	20	64
Latvia (**III**)	116	26	20	54
Lithuania (**IV**)	71	20	11	69

the association between metalaxyl resistance and year remained statistically non-significant (p = 0.21, Figure 3, **III**). The distribution of metalaxyl resistance over different agricultural management practices was not dependent on year either (p = 0.13, **III**). A similar result, with differences in metalaxyl resistance between sampling years statistically non-significant was found in the Lithuanian population of *P. infestans* (Chi-square = 4.43, df = 4, p = 0.35, **IV**).

Response to metalaxyl resistance did not differ significantly between sampling sites in Estonia in 2013 (Chi-square = 24.83, df = 16, p = 0.07, **II**). At the same time, in the Lithuanian population of the pathogen, significant differences were present between metalaxyl resistance and sampling sites (Chi-square = 43.64, df = 19, p = 0.001, **IV**). There were more intermediate isolates in northern and eastern sites and sensitive isolates were more often present in central and south-western regions, but the differences between regions were statistically non-significant (Chi-square = 11.36, df = 6, p = 0.078, **IV**).

The distribution of metalaxyl resistance was similar over different agricultural management practices in the Latvian population of *P. infestans* (p = 0.66, **III**). Metalaxyl resistant isolates were not present in five large scale conventional fields, in one small scale conventional field and in one organic field – in the total from seven fields. Sensitive strains were missing from one organic and three large scale conventional fields – in the total from 4 fields. In Lithuania, the association between metalaxyl resistance and different agricultural management practices remained also non-significant (Chi-square = 3.59, df = 4, p = 0.46), but there was a slightly higher frequency of resistant isolates from field trials than from large and small conventional fields (**IV**).

The association between metalaxyl resistance and mating types was not statistically significant in Estonia in 2004–2005 (Chi-square = 3, df = 1, p = 0.083, **I**), whereas 65% percent of the metalaxyl resistant strains were A1 mating type, 30% were A2 mating type and 5% were self-fertile. The association was also non-significant in the 2013 samples (Chi-square = 1.11, df = 2, p = 0.57) (**II**). In the Latvian population, the proportion of sensitive isolates was highest among A1 mating type and the resistant isolates were more present among A2 mating type (Figure 4, **III**), although this association was statistically non-significant (p = 0.09), whereas after leaving out the self-fertile mating type (3 isolates tested for metalaxyl resistance), the association become significant (p = 0.04, **III**). Testing the association by year resulted in a significant difference only in 2012 (p = 0.006 and 0.003, respectively considering and not the self-fertile mating type). Testing the distribution of metalaxyl resistance over years by mating types resulted in statistically significant change for mating type A1 (p = 0.014) but not for mating type A2 (p = 0.56) (**III**). In the Lithuanian population of *P. infestans* the association between metalaxyl resistance and mating type was present (Chi-square = 11.36, df = 4, p = 0.024, **IV**), furthermore out of 39 A1 mating type isolates, three were metalaxyl-resistant, three intermediate in resistance and 33 sensitive isolates and out of 29 A2 mating types, the numbers were 10, 5 and 14, respectively. One resistant isolate and two sensitive isolates were found out of three self-fertile isolates (Figure 3B, **IV**).

5.3. Virulence

The overall frequencies of resistance genes R1–R11 are presented in Table 5. All 11 known virulence factors were found among all the populations. The most frequent was virulence against factors R1, R2, R3, R4, R7, R10 and R11 and virulence was rare against R5, R8 and R9 similarly in all studied populations (Figure 1, **I**; Figure 1, **II**; Figure 5, **III**; Figure 4, **IV**, respectively).

The two most common races made up 70% of the isolates tested in Estonia in 2004–2005 (Table 5, **I**), whereas, in 2013, the most common races were 1.2.3.4.5.6.7.8.10.11, 1.2.3.4.6.7.8.10.11, 1.2.3.4.7.10.11 and 1.2.3.4.7.8.10.11, which formed 46% of the population (Table 5, **II**). The four most common pathotypes in the Latvian population – 1.2.3.4.7.10.11, 1.2.3.4.6.7.10.11, 1.2.3.4.5.6.7.8.9.10.11 and

Table 5. The frequencies of resistance genes R1–R11 in *P. infestans* populations (**I**, **II**, **III**, **IV**).

Country	Isolates tested	Frequencies of virulence factors (%)										
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Estonia (**I**)	196	93	35	97	94	10	29	97	10	2	95	96
Estonia (**II**)	104	90	74	81	81	28	43	85	50	11	92	88
Latvia (**III**)	156	87	76	82	80	27	58	85	32	24	85	88
Lithuania (**IV**)	70	84	79	73	76	26	79	90	23	16	86	93

1.2.3.4.6.7.8.10.11 – covered 33.6% of isolates (**III**), and this percentage was really similar to Lithuanian population results, where the most frequently occurring races were: 1.2.3.4.6.7.10.11, 1.2.3.4.5.6.7.8.9.10.11, 1.2.3.4.7.10.11 and 1.2.3.4.5.6.7.10.11 representing 34% of the isolates (**IV**).

In 2004–2005 in Estonia, 49 different races were identified (**I**), and, in 2013, the number was similar, being 42 (**II**), whereas 26 races were unique and found only once (Table 5, **II**). In the Latvian population, the number of different pathotypes was 69, whereas in the Lithuanian population, 38 different pathotypes (Table 3, **IV**) were recorded and 36% of the pathotypes were found only once and in 2011 – half of the pathotypes were unique (Table 3, **IV**).

The average number of virulence factors per isolate in the Estonian population (2004–2005) was 6.7 (Table 5, **I**), being somewhat lower than in 2013 – 7.2, varying between fields from 5.6 to 9.0 (**II**). Interestingly, in Latvian and Lithuanian populations, the value was exactly the same – 7.2 (**III**, **IV**), whereas this varied between sites in Lithuania from 5.2 to 10.3, whereas complex races were more often present among isolates collected from small scale fields (7.5) than from trial fields (**IV**).

The overall normalized Shannon diversity index values were 0.38 (2004–2005) and 0.69 (2013) in Estonia and varied in 2013 between potato fields from 0.43 to 0.95 (Table 4, **II**). Slightly higher values were calculated in the Latvian population, being 0.73 (**III**) and in the Lithuanian population of the pathogen, being 0.79 (**IV**).

Additional analyses were carried out for the Latvian population of the pathogen and the results are presented in the following passages. The R-genes interaction effects with year and field type were statistically significant (both $p < 0.001$), showing that difference between virulence to specific R-genes in different years and field types exists. Relationships between virulence to different R-genes and metalaxyl resistance were statistically non-significant. In relationship with mating types, four R-genes (R3, R7, R9 and R11) had statistically significantly different proportions of virulence, but only two of these relationships remained statistically significant after correction for multiple testing (R3: $p = 0.011$, R7: $p = 0.003$) resulting in higher virulence among the A1 mating type (**III**).

The hierarchical cluster analysis of virulence against R-genes brought out two groups of genes – one group, which consists of the rarely found R-genes (R5, R8 and R9) and the second group that consists of all the other R-genes (Figure 6, **III**). In the second group the closest R-genes to the first group are R6 and R2 with intermediate frequency of virulence. This outcome shows that if an isolate is virulent against one rare R-gene then it is presumably virulent against another rare R-gene and also the virulence against more common R-genes tend to exist at the same time (**III**).

The principal component analysis performed on isolates, collected from Latvia showed that first two principal components describe 44.7% of the total variability of virulence against different R-genes (**III**). The first principal component identifies less and more virulent isolates (Figure 7, **III**) and the second pattern recognizes isolates virulent against less and more common R-genes. The virulence in common was more frequent in organic fields and among A1 and metalaxyl-resistant isolates and less frequent in 2010 as indicated by the values of the analysis of principal components. The virulence against rarer R-genes appeared more often in organic fields and in 2011, less often in breeding fields and among intermediate metalaxyl-sensitive isolates (Figure 7, **III**).

Significant difference in the prevalence of virulence factors R2, R5, R8 and R9 was found between sampling years in Estonia, 2004–2005 (R2: Chi-square = 10.95, $df = 1$, $p = 0.0009$; R5: Chi-square = 9.38, $df = 1$, $p = 0.0022$; R8: Chi-square = 16.03, $df = 1$, $p < 0.0001$; R9: Chi-square = 5.55, $df = 1$, $p = 0.019$). After applying the Bonferroni correction, only differences in R2, R5 and R8 remained statistically significant (**I**). In the Latvian population of the pathogen, a statistically significant dif-

ference was present ($p < 0.001$) in virulence to specific R-genes (Figure 5, **III**). The overall virulence varied between years and was statistically significantly different ($p < 0.001$) being lower in 2010 and higher in 2012. The proportions of isolates virulent to R6 and R9 were statistically significantly different at 2011 and 2012 ($p = 0.013$ and $p = 0.001$, respectively) as appeared from the results of Fisher exact tests, which were followed by Holm correction for multiple testing, whereby the resistance against both R6 and R9 was more common in 2011 (**III**). A statistically significant difference, within the two sampling years in Lithuania, was found in the prevalence of virulence factors ($F_{(10,175)} = 28.36, p < 0.001$). Relatively rare were virulence factors 9 (2011: 8.6 ± 5.4%; 2012: 20.8 ± 16.3%), 5 (2011: 15 ± 6.7%; 2012: 24.33 ± 10.5%) and 8 (2011: 19.8 ± 8.8%; 2012: 15.3 ± 11.1%) (Figure 4, **IV**), but the differences between virulence factors and sampling years in Lithuania were non-significant ($F_{(1,175)} = 2.44, p < 0.12$). In 2011, the normalized Shannon diversity index reached 0.87 in Lithuania and in 2012 it was lower – 0.75, but there was no statistically significant difference between values between studied years ($F_{(1,3)} = 4.22, p = 0.18$).

Differences in virulence factors between field sites were non-significant in the Estonian population, sampled in 2013 ($F_{(8,80)} = 2.64, p = 0.11$) (**II**). However, in Lithuania, the association was statistically significant ($F_{(16,160)} = 3.88, p < 0.0001$) (**IV**), though no significant differences were found in virulence factors between different regions ($F_{(3,170)} = 0.41, p = 0.75$). There were no significant differences in the values of the normalised Shannon diversity index between different regions ($F_{(1,3)} = 0.54, p = 0.69$) (**IV**).

The virulence against R6 and R9 was different at different agricultural management practices in Latvia ($p = 0.001$ and $p < 0.001$, respectively), whereby the resistance against both R6 and R9 was more common in organic fields (**III**). The overall virulence in Latvian ($p = 0.88$, **III**) and Lithuanian ($F_{(2,170)} = 1.74, p = 0.18$, **IV**) populations remained non-significant between agricultural management practices.

6. DISCUSSION

6.1. Characterisation of *Phytophthora infestans* Baltic populations

In this thesis, studies in Baltic region populations of the phenotypic characteristics of *P. infestans* isolates, collected from Estonia in 2004–2005 and in 2013, and from Latvia and Lithuania in 2010–2012, are reported and results compared with *P. infestans* populations from other European countries. All three Baltic countries belong to the same European region and therefore it is reasonable to compare the pathogen populations in Latvia and Lithuania, where only little data was available before this study, with Estonian populations of the pathogen, where the research have been going on for longer period of time, to see the population structure in the larger frame of the Baltic context. The phenotypical markers used for characterisation were mating type, metalaxyl resistance and virulence.

6.1.1. Mating type

The mating type proportions (A1:A2) in Estonia (57:41) (2004–2005), Latvia (53:43) and Lithuania (50:45) are close to 1:1 (**I, III, IV**). The variance in mating type frequencies in 2013 in Estonia (**II**) is higher (71:29), but the increase of A1 mating type proportion is probably temporary and influenced by year, since lower proportions of A1 mating type have been common in earlier population studies in Estonia (Runno-Paurson *et al.*, 2010, 2013b, 2014), and there have been fluctuations in the frequencies of mating types in Estonia before (Runno-Paurson *et al.*, 2011, 2012). These results show that the Baltic countries populations, described in this thesis, are generally comparable.

The ratio of two mating types in the pathogen populations in Estonia, Latvia and Lithuania is almost perfect for sexual reproduction and formation of oospores to take place (Turkensteen *et al.*, 2000). Furthermore, investigation into the populations of the pathogen in Estonia, Latvia and Lithuania proved the presence of both mating types together in all three countries and almost all the studied sites (**I, II, III, IV**). This result provides circumstantial evidence that the pathogen can reproduce sexually in Baltic potato late blight populations, moreover, equal pecentages of A1 and A2 mating types on the same fields lead to greater quantities of oospore formation (Yuen, Andersson, 2013). It is clear that the mating

type frequency according to the studies carried out in the three countries indicates continuous sexual reproduction in the populations of *P. infestans* in the Baltic countries, which leads to earlier infections in the potato fields and soil contamination with long living oospores (**I**, **II**, **III**, **IV**).

Formation of oospores does not automatically mean that these are the inoculum for the next season, because oospores have to survive first and after winter must be able to infect new plants effectively; moreover germination of oospores is complicated even in a laboratory situation (Yuen, Andersson, 2013). Still, oospores in the soil present a threat to widely used preventative strategies against potato late blight (Yuen, Andersson, 2013), due to the fact that oospore derived infections start earlier (which is also happening in the Baltic countries) and are not immediately visible, since the infection starts to develop on the lower leaves which may be hidden by upper leaves. Therefore, it is hard to predict the best time for the first fungicide application and the potential damage might be larger. Many studies have shown that oospores do not need a host plant to survive in the soil (Drenth *et al.*, 1995, Turkensteen *et al.*, 2000), and that the cool temperatures during the winter, in the Nordic climate conditions, create a suitable environment for preserving oospores in the soil for 3–4 years (Cooke *et al.*, 2011; Hannukkala, 2012); this limits the frequency with which potato can be cultivated in the same field (Yuen, Andersson, 2013).

Similar results, concerning the ratio of A1 and A2 mating types, have been reported from Finland, Denmark, Norway and Sweden (Lehtinen *et al.*, 2008; Hannukkala, 2012), Poland (Chmielarz *et al.*, 2014) and the north-western part of Russia (Statsyuk *et al.*, 2013). Results, where the incidence of A2 mating type is higher than the incidence of A1 mating type, have been reported from Ireland, where in 2009 and 2010, the proportions of A2 mating type were 60% and 75%, respectively (Cooke *et al.*, 2012). Also Gisi *et al.* (2011) reported an increased level of A2 mating type frequency from France, Switzerland, Belgium and Denmark in 2006–2007.

One possible reason for the difference in the frequency of A2 mating type between the two investigation years in Estonia, where the prevalence of A2 was lower in 2004 by 26% than in 2005, might have been differences in sample size. The proportion of mating types is influenced by the total number of isolates collected, furthermore, the smaller the number, the

greater the impact. For example, if one site from which several isolates have been collected has been invaded by one aggressive strain (whether A1 or A2), it overrules the result from several other sites, from which only one or two isolates have been taken. Another factor might have been the choice of cultivar or pathogen adaptation ability. An increase of A2 mating type over time was recently reported in a French population, where the low proportions of A2 (<15%) suddenly rose to more than 75% in 2007–2008 (Mariette *et al.*, 2015). There have been fluctuations, which favour A2 mating type also in Poland (Chmielarz *et al.*, 2014). The association between the proportion of mating types and sampling sites in Estonia (2013, **II**) and Lithuania (**IV**) might have been influenced by differences in local growth conditions as well as by sample size (discussed earlier).

Sustainable potato production is threatened by the spread of aggressive clonal lineages, which are able to break through the late blight resistance of various potato varieties (Cooke *et al.*, 2012). An additional risk factor may be increased oospore production, which reduces the effect of crop rotation, if it remains short (Lehtinen *et al.*, 2008). The potato late blight pathogen can adapt well in areas where potato production is disunited (since small scale conventional farms are predominant, chemical control of potato late blight is not always affordable, crop rotation systems could be more effective) and sexual reproduction is occurring frequently (Brylinska *et al.*, 2016). Previous description matches also with the situation in the Baltic countries as the populations are variable in genotype (Runno-Paurson *et al.*, 2016), possibly due to frequent sexual reproduction. Diverse populations are more resistant to invasions of clonal genotypes (Fry *et al.*, 2015) and this might indicate that the potato production in Baltic countries does not have to face invasion of aggressive clonal lineages yet, nonetheless, the potato producers in Baltic countries are challenged by frequent oospore formation and therefore early outbreaks of the disease. The results of mating type analyses in the studies presented in the thesis support the evidence that the northern population of late blight pathogen in Europe (Yuen, Andersson, 2013) together with Baltic countries populations can be called the second centre of sexual reproduction of *P. infestans*.

6.1.2. Metalaxyl resistance

The proportion of metalaxyl-resistant isolates in Estonia (2004–2005) was relatively high, present in nearly half (49%) of the analysed samples (**I**). Still, the results in Estonian (2013), Latvian and Lithuanian populations of the pathogen during 2010–2013 were quite similar with domination of metalaxyl-sensitive isolates (**II**, **III**, **IV**). The high incidence of metalaxyl-resistant isolates may have been caused by the intense metalaxyl treatments, due to the early and rapid development of the disease in the fields, induced by the favourable weather conditions for the pathogen in these years. Metalaxyl-resistant strains dominated the pathogen population in Lithuania in the 1990s (Valskytė, 2000). A high frequency of metalaxyl-resistant strains was also found in the pathogen populations in Ireland (2009), which was most likely connected to the dominant aggressive lineage 13_A2, which broke host resistance fast (Cooke *et al.*, 2012). In France (2007), the percentage of metalaxyl-resistant strains was as high as 80 (Duvauchelle *et al.*, 2009) and also dominated the population in the United Kingdom in 2006–2007 (Gisi *et al.*, 2011).

A major change in response to metalaxyl has taken place in Estonian populations of the pathogen, where the proportion of resistant isolates decreased to 16% and that of sensitive strains increased to 64% (**II**). An analogous change occurred in the Lithuanian population of the pathogen, where in 1990 the percentage of metalaxyl-resistant isolates was 98%, due to intense application of metalaxyl-based fungicides on the fields, and after 7 years, the percentage dropped to 23% in 1998 (Valskytė, 2000). In 1990–1995, metalaxyl-based products were the only systemic fungicides of the phenylamide group in commercial use (Valskytė, 2000). However, seasonal fluctuations in response to metalaxyl are not extraordinary in *P. infestans* populations (Cooke *et al.*, 2011).

The higher frequency of metalaxyl-sensitive strains may indicate low usage of fungicides on the fields from which the isolates were collected. In Latvia and Lithuania, the proportion of small-scale potato productions is high – 97% and 93%, respectively (Cudere, 2008; Ronis *et al.*, 2007) and, as fungicides are expensive, their application is a measure of last resort. Still, the fact that metalaxyl-sensitive isolates prevail in Estonian, Latvian and Lithuanian populations of *P. infestans*, suggests that metalaxyl-based products can be used effectively as one of the methods for controlling late blight disease. Montes *et al.*, (2016) point out that

despite the fact that *P. infestans* can develop insensivity towards metalaxyl, it is still very useful in fighting against late blight and it still finds a use in most regions. Metalaxyl-based products are also widely used in Estonia (Runno-Paurson *et al.*, 2014), but for example in Finland, similar products comprise less than 10% of all fungicides used to prevent late blight development (Hannukkala *et al.*, 2007), and at the same time metalaxyl-M is not in commercial use in Denmark (Lehtinen *et al.*, 2008).

The dominance of metalaxyl-sensitive strains in the pathogen populations in the Baltic countries (**II**, **III**, **IV**) concur with the populations in the Nordic countries – Norway, Sweden, Finland and Denmark (Lehtinen *et al.*, 2008) and in Poland, where the proportion of metalaxyl-sensitive strains reached 75% in 2007–2009 (Chmielarz *et al.*, 2014) also the Moscow region of Russia (Statsyuk *et al.*, 2013), Belarus (Pobedinskaya *et al.*, 2011) and Czech Republic (Mazáková *et al.*, 2011). More than 50% of metalaxyl-sensitive strains have been reported also from Northern Ireland in 2011 and 2013 (<eucablight.org>).

The fact that no important connection between metalaxyl-resistance and study years was found from any of the countries' populations, probably indicates a stable and solid management with fungicides these years. This situation is similar to that observed in the 1990s, where metalaxyl resistance did not fluctuate considerably (Gisi, Cohen, 1996). If metalaxyl-based products are used properly, following the instructions, then the possibility for metalaxyl-resistant strains to become dominant in the pathogen population is low (Runno, Koppel, 2006). Nevertheless fluctuations in metalaxyl resistance, from season to season, are considered normal (Gisi, Cohen, 1996) and linked directly on how metalaxyl is used (Dowley *et al.*, 2002). In general, the response to metalaxyl did not depend on the site, although the association between those factors was important in Lithuanian population (**IV**).

The relationship between metalaxyl resistance and agricultural management practices remained non-significant in all studied countries (**II**, **III**, **IV**), a result that concurs with the findings from Estonia 2004–2007 (Runno-Paurson *et al.*, 2010). Probably management practices do not always affect the populations since organic and conventional fields are mixed within a region and *P. infestans* strains can easily migrate from one field type to another. The slightly higher percentage of metalaxyl-resistant strains in two specific conventional fields in Estonia (LSC – Verioramõisa

and SSC – Võnnu, Table 3, **II**), is probably connected with the fact, that in conventional farming systems, the growers are using metalaxyl-based fungicides to control late blight at the beginning of disease infection, and the notably high percentage (36%) of metalaxyl-resistant strains in the organic field (**II**) can be explained with the seed potato production field within reach and possible inoculum spread by wind.

Opposite results also have been reported from Estonia, where significant differences in metalaxyl-resistance between agricultural management practices were found (Runno-Paurson, 2010). A recent study from Poland also confirmed that cultivation system may influence population structure where metalaxyl-resistant and intermediate isolates dominated the region (Młochów) and the majority of samples came from large scale production fields (Brylinska *et al.*, 2016). In Latvia, the distribution of metalaxyl resistance didn't differ over agricultural practices (**III**) and in Lithuanian populations, there was only a slight indication of more resistant isolates on the field trials, compared to large and small scale conventional fields (**IV**).

In Estonian and Latvian populations of *P. infestans*, the relationship between metalaxyl resistance and mating types remained statistically non-significant (**I**, **II**, **III**), whereas in the Lithuanian population of the pathogen, a strong association between the two phenotypic markers was found, the relationship between A2 mating type and metalaxyl-resistant strains was significant (**IV**). During the 1990s, resistance to metalaxyl was related mostly with A1 mating type (Hermansen *et al.*, 2000), but a strong relationship between A2 mating type and metalaxyl-resistant strains has been found in several studies in recent years (Cooke *et al.*, 2011). Nevertheless, no genetic link between mating type and metalaxyl resistance has been discovered and the connection between metalaxyl-resistant strains and A2 mating type may be coincidental (Gisi *et al.*, 2011).

6.1.3. Virulence

All known virulence factors were found from all studied countries (**I**, **II**, **III**, **IV**); the most common virulence being R1, R2 (**II**, **IV**), R3, R4, R6 (**IV**), R7, R10 and R11 (**I**, **II**, **III**, **IV**). The rarest virulence factors in all Baltic countries were R5, R8 and R9 (**I**, **II**, **III**, **IV**). Virulence factor R2 was rare as well; this has also been reported from Poland, Denmark, Fin-

land, Norway and Sweden (Lehtinen *et al.*, 2008; Chmielarz *et al.*, 2014) and previously from Estonia (Runno-Paurson *et al.*, 2009, 2010). The frequency of the R2 virulence factor is undergoing change, for example in Estonia, its frequency has increased over the years (**II**), and in Latvia is relatively high, with more than 70% of the isolates tested able to overcome the R2 virulence factor (**III**). This result over the three Baltic countries shows clearly, that R-genes, used as a preventative method against late blight, may not be very useful; the pathogen has the ability to overcome the resistance, created with these resistance genes (Yuen, Andersson, 2013). There is a possibility that the rarely found R-genes (R5, R8, R9), combined with other preventative and protective methods against the disease, may be useful, but if varieties containing them became commercial and widely used, the resistance would probably break down fast.

The race structure in the Baltic countries was highly diverse and complex (**I**, **II**, **III**, **IV**). The number of different pathotypes among isolates tested was quite high in all Baltic countries (**I**, **II**, **III**, **IV**). The proportion of unique races (found only once) was in Estonia 26% (2013), in Latvia 35% and in Lithuania 36%. Earlier studies in Estonia have reported an even higher proportion of unique races – nearly half of the identified pathotypes being found only once (Runno-Paurson *et al.*, 2009).

In all Baltic countries there were isolates in the pathogen's population, which were able to overcome all the virulence factors with the race 1.2.3.4.5.6.7.8.9.10.11 (**I**, **II**, **III**, **IV**). In Finland (1997–2000), only one isolate with the same race was found (Lehtinen *et al.*, 2007). One of the most common races in Baltic countries was 1.2.3.4.6.7.10.11 (**I**, **III**, **IV**), with the exception of the Estonian population in 2013, where the most common race was 1.2.3.4.5.6.7.8.10.11 (**II**). This result is not common for other European populations of the pathogen (Hermansen *et al.*, 2000, Lehtinen *et al.*, 2007, Lehtinen *et al.*, 2008, Runno-Paurson *et al.*, 2009, 2014; Hannukkala 2012). The dominant race in Europe and in the Nordic countries 1.3.4.7.10.11 (Hermansen *et al.*, 2000; Lehtinen *et al.*, 2008; Hannukkala, 2012; Runno-Paurson *et al.*, 2014) was found four times from Estonian (2013), five times from Latvian and twice from Lithuanian populations of the pathogen (**II**, **III**, **IV**). Nevertheless, in 2004–2005, in the Estonian population, it was the dominant race (**I**), which shows another change that has taken place in the Estonian population of *P. infestans* next to the metalaxyl resistance. Complex races have dominated in Estonia over the years (Runno-Paurson *et al.*, 2009, 2010,

2013, 2014) and are common in Russia (Statsyuk *et al.*, 2013) as well as in Poland (Chmielarz *et al.*, 2014).

The mean number of virulence factors (Black's differentials) per isolate was high in all studied Baltic populations (7.2) (**II**, **III**, **IV**) showing that complex races are common to these populations. However, the Estonian population of *P. infestans* in 2004–2005 differed from newer results where the number was slightly lower (6.7) (**I**) and therefore similar to the results reported from Nordic countries (Lehtinen *et al.*, 2008). Results of Baltic countries in 2010–2013 are similar to those from Eastern European populations (Śliwka *et al.*, 2006; Statsyuk *et al.*, 2013; Runno-Paurson *et al.*, 2014) as well as to previous studies in Estonia (Runno-Paurson *et al.*, 2012, 2014).

The high pathotype diversity in the *P. infestans* populations in the studied Baltic countries, is supported by the values of the normalized Shannon diversity indexes, being lowest in Estonia in 2004–2005 – 0.38 (**I**). Still, another change has occurred in the Estonian population of the pathogen, and the Shannon diversity index has increased to 0.69 (**II**) in 2013. In Latvia and Lithuania, the values were slightly higher – 0.73 and 0.79, respectively (**III**, **IV**). The high pathotype diversity is probably linked to the fact that both mating types (A1 and A2) occurred in the same fields at the same time and therefore the high diversity may be the result of sexual reproduction, which enhances the diversity of the pathogen in the population (Mayton *et al.*, 2000). Another possible source of high phenotypic diversity might be that contaminated seed potatoes are used in production (Runno-Paurson *et al.*, 2013a), because in the cooler climates, where the temperature drops below 0 °C, the survival of the pathogen over winter is limited, and therefore infected seed tubers and oospores are a very important source of inoculum (Widmark *et al.*, 2007; Runno-Paurson *et al.*, 2013a).

The differences in the prevalence of virulence factors in the growing season (**III**, **IV**), between different study years (**I**, **III**), and sites (**IV**) are probably influenced by the choice of cultivars, occurrence of sexual reproduction and presence of agressive strains of the pathogen in the populations. For example, in Estonia, growers often choose to plant Western-European early varieties, instead of locally bred and more resistant varieties. Runno-Paurson *et al.*, (2013a) points out several reasons for that, like the chosen varieties provide high and early yield, which can be marketed

fast. Furthermore, there is not enough seed material of local varieties (for large scale productions) and together with the relatively high resistance in variety, a trait such as 'late maturity' comes along. Small scale growers often use seed from their own propagation and this material is often not free of the tuber blight. Sexual reproduction diversifies the populations of the pathogen and in certain years some aggressive strains of *P. infestans* might also be present, which can influence the prevalence of various virulence factors (Cooke *et al.*, 2012).

7. CONCLUSIONS

This study confirmed high phenotypic variability among Estonian, Latvian and Lithuanian populations of *P. infestans,* and it can be concluded that populations of this pathogen in the Baltic countries are diverse and highly complex.

Mating type ratio in Estonian, Latvian and Lithuanian populations of *P. infestans*, based on this study, was close to 1:1 throughout the three studied years, furthermore, mating types A1 and A2 were present in the same fields at the same time, indicating possible sexual reproduction in these pathogen populations. There is a high risk for possible soil contamination with long-living oospores on the fields, where the samples were collected, which emphasizes the importance of a rigorous crop rotation system.

The results in Estonian, Latvian and Lithuanian populations of the pathogen, concerning the metalaxyl-resistance, during 2010–2013 were quite similar with domination by metalaxyl-sensitive isolates. Therefore metalaxyl-based products may be useful in managing the late blight disease in the Baltic countries.

All known virulence factors were found from all studied countries, the most common virulence factors were R1, R2 (**II**, **IV**), R3, R4, R6 (**IV**), R7, R10, R11. The rarest virulence factors in all Baltic countries were R5, R8 and R9. The specific resistance genes R1–R11 are probably not very useful for future potato breeding, due to the pathogen's ability to break through the resistance provided by r-genes.

Differences derived from time, site and agricultural management practices are present within the populations of *P. infestans* in the Baltic countries. These are most likely influenced by sample sizes, local management practices, choice of cultivars etc. Furthermore, diverse populations influence the population structure as well.

In general, the populations of *P. infestans* are similar in Estonia, Latvia and Lithuania. Minor differences can be considered normal as the populations are highly diverse and the pathogen is continously changing.

Issues requiring further research:

Since *P. infestans* has the ability to adapt to different climatic conditions as well as to survive in unfavourable environmental conditions, it is very important to monitor the pathogen each year in the Baltic countries, to be aware of the changes taking place in the populations of the pathogen. We need knowledge of the changes to be able to control the disease on the fields. Furthermore, the world faces global warming and we do not yet know how this will influence pathogen populations, we can only quess, therefore continuous phenotypic and genotypic characterisation of *P. infestans* is important.

There is information about both mating types in the same fields from all Baltic countries. Oospores are being formed, but it is important to carry out research to find out the importance of oospores in causing early infections of the potato fields. There is no clear information about the proportion of oospore derived infections in Estonia, Latvia and Lithuania. This knowledge would be helpful in developing more effective methods of fighting against the disease.

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KARTULI-LEHEMÄDANIKU TEKITAJA *PHYTOPHTHORA INFESTANS* BALTIKUMI POPULATSIOONIDE FENOTÜÜBILINE ISELOOMUSTAMINE

Kartulikasvatuses suurimaid saagikadusid põhjustav kartulihaigus on kartuli-lehemädanik, mida tekitab seenetaoline organism *Phytophthora infestans* (Mont.) de Bary. See patogeen on tuntud kogu maailmas ja kuigi tema tundmaõppimine ning tõrjealased intensiivsed uurimistööd on kestnud pikka aega, tekitab patogeen endiselt märkimisväärseid saagikadusid ega allu keemilisele tõrjele. Regulaarne ja süstemaatiline fungitsiidide kasutamine aitaks küll haiguse arengut pidurdada (Cooke *et al.*, 2012), kuid peamiselt preparaatide kalliduse tõttu ei ole enamasti võimalik komplekset tõrjet rakendada.

Kartuli-lehemädanik on tõsine probleem Eesti, Läti Leedu kartulipõldudel. Keskmine saagikadu Baltikumis jääb 15–30% piiridesse (Runno-Paurson *et al.* 2010) kuid patogeenile soodsates tingimustes võivad saagikaod olla kuni 50% (Ronis, Tamošiunas, 2005). Kartuli tootmise pindala on viimase kümne aastaga Eestis vähenenud 59% ning kogupindalast 57% kuulub suurtootjatele. Kasvatustehnoloogiad on Lätis ja Leedus küllaltki sarnased, Lätis toodetakse 97% kartulist alla 1ha suurustel põldudel, Leedus aga toodetakse 90% kartulist alla 5 ha põldudel. Enamus tootjaist kasutab väga vähe fungitsiide (Ronis, Tamošiunas, 2005). Varasemad uuringud on näidanud, et Eestis pole võimalik ilma fungitsiide kasutamata saada suurt ja kvaliteetset saaki (Koppel, 1997).

Taimekaitsevahendite efektiivne kasutamine võib tagada tootjale teatud perioodil küll kõrgema kvaliteediga suurema saagi, kuid ei taga kartuli-lehemädaniku probleemile lõplikku lahendust, patogeen on pidevas muutumises ning seetõttu uurijatest ees. Näiteks on viimastel aastakümnetel haigustekitaja populatsioonides toimunud suured muutused, kus nn. „vana" A1 paarumistüübiga *P. infestans* populatsioon on segunenud või asendunud uue, mitmekesise ja agressiivse A2 paarumistüübiga. Need paarumistüübid võivad esineda koos ning patogeen on võimeline suguliselt paljunema (Fry *et al.*, 1993; Cooke *et al.*, 2011), mille tulemusena moodustuvad vastupidavad paksukestalised oospoorid, mis võimaldavad patogeenil pikka aega mullas ilma peremeestaimeta eluvõimelistena püsida (Turkensteen *et al.*, 2000).

Suguline paljunemine muudab populatsiooni struktuuri geneetiliselt mitmekesisemaks (Chmielarz *et al.*, 2014). Uued genotüübid populatsioonis mõjutavad haiguse arengut (Lees *et al.*, 2008) ning need muutused on kartulipõldudel selgesti nähtavad: nakkus algab varem, on intensiivsem ning vajadus fungitsiidide järele suureneb (Runno-Paurson *et al.*, 2014). Varem meie aladel esinenud A1 paarumistüübi puhul algasid haiguspuhangud põldudel nüüdsest kuu aega hiljem ja ei olnud nii agressiivsed (Runno-Paurson *et al.*, 2013a). Samuti on täheldatud, et sugulise paljunemise puhul on populatsioonis kasvanud keeruliste kombineeritud virulentsusfenotüüpide osakaal (Lehtinen *et al.* 2007; Runno-Paurson *et al.*, 2009, 2014; Hannukkala, 2012; Statsyuk *et al.*, 2013). Kuigi tuleb ette veel *P. infestans*'i populatsioone, kus on väike arv genotüüpe ja suguline paljunemine on see siiski pigem harvaesinev (Gisi *et al.*, 2011; Cooke *et al.*, 2012; Li *et al.*, 2012).

Senine informatsioon haigustekitaja Läti ja Leedu populatsioonide kohta oli vähene ja sageli ka vananenud. Lätis identifitseeriti *P. infestans*'i rasse aastatel 1974–1990 ja A2 paarumistüübi esmasleid oli seal 1987 aastal (Bebre *et al.*, 2004). Viiteid oli ka oospooride tekke kohta Läti kartulipõldudel, kuid puudus informatsioon A1 ja A2 paarumistüüpide jagunemise kohta (Bimšteine, 2008). Informatsioon Leedu populatsioonide kohta piirdus metalaksüüli resistentsuse katsetega, mis viidi läbi 1990–1996 (Valskyté, 2000). Informatsiooni A2 paarumistüübi ja haigustekitaja virulentsuse kohta pole avaldatud.

Lähtudes nendest probleemidest oli doktoritöö töö peamiseks eesmärgiks võrrelda *P. infestans*'i populatsioone Eestis, Lätis ja Leedus.

Selleks tuli selgitada:
1) milline on fenotüübiline varieeruvus patogeeni Eesti (**I, II**), Läti (**III**) ja Leedu (**IV**) populatsioonides, võttes hindamise aluseks paarumistüübid, metalaksüüli-resitentsus ja virulentsus ning saadud näitajate põhjal hinnata kui palju erinevate maade populatsioonid üksteisest erinevad;

2) kas paarumistüüpide suhe Läti ja Leedu haigustekitaja populatsioonides osutab sugulise paljunemise võimalustele (**III, IV**);

3) kas Baltikumi *P. infestans*'i populatsioone mõjutavad aeg (**I, II, III, IV**), koht (**II, IV**) ja viljelusviisis (**II, III, IV**) ?

Hüpoteesid:

- *P. infestans*'i populatsioonid Eestis, Lätis ja Leedus on väga mitmekesised ja keerulised.

- Paarumistüüpide suhe *P. infestans*'i Eesti, Läti ja Leedu populatsioonides viitab sugulise paljunemise võimalustele.

- Baltikumi *P. infestans*'i populatsioonid on üksteisega sarnased.

- *P. infestans*'i populatsioonides esinevad ajast, kohast ning viljelusviisist tingitud erinevused.

Kolmest Balti riigist – Eestist aastatel 2004–2005 (Tabel 2) (**I**) ja 2013 (Tabel 1, **II**), Lätist aastatel 2010–2012 (Tabel, **III**) ning Leedust aastatel 2010–2012 (Tabel 1, **IV**) – koguti *P. infestans*'i isolaadid ning määrati patogeeni paarumistüübid, metalaksüüli-resistentsus ning virulentsus. Enamus uuritud põldudest olid tavatootmispõllud – väiketootmispõllud (27) ja suurtootmispõllud (23), väike osa kuulus mahe- (8), sordiaretus- (2) ja katsepõldude (6) koosseisu. Vaatluse all olnud väiketootmispõldudel oli tootjatel kasutada ebakindla kvaliteediga seemnekartul ja kartuli-lehemädaniku tõrjumiseks ei kasutatud fungitsiide või kasutati neid ebaregulaarselt. Suurtootmispõldudel kasutati sertifitseeritud seemnekartulit ja haigustõrjet tehti regulaarselt ning järgiti viljavaheldust.

Kartulitaimed, millelt nakatunud lehed korjati, valiti põllu servadest juhuslikelt kaugustelt. Nakatunud kartulitaimedelt valiti ühe nakkuslaiguga lehed. Kogutud isolaadid viidi puhaskultuuri (**I**, **II**, **III**, **IV**). Paarumistüüpide määramiseks kasvatati uuritavat isolaati 10–18 päeva 16 °C juures kasvukambris Petri tassis rukki söötmel koos vastava testerisolaadiga (A1 või A2) (**I**, **II**, **III**, **IV**). Metalaksüüli-resistentsuse määramiseks kasutati Hermansen *et al.*, (2000) kirjeldatud ning Runno-Paurson *et al.*, (2009) modifitseeritud 'ujuva lehe meetodit' (**I**, **II**, **III**, **IV**). Spetsiifiline virulentsus määrati nakatades resistentsusgeene R1–R11 sisaldavaid diferentsiaatorsorte (Malcolmson, Black, 1966) (**I**, **II**, **III**, **IV**).

Käesoleva doktoritöö tulemused kinnitasid varasemates töödes saadud tulemeid (Runno-Paurson *et al.*, 2009, 2010, 2011, 2012, 2014), et *P. infestans*'i Eesti populatsioonis on suur fenotüübiline varieeruvus (**I**, **II**). Analüüsiti ka neid muutusi, mis haigustekitaja Eesti populatsioonis

on viimasel kümnendil aset leidnud. Selgus, et kõige tõenäolisemalt just sugulise paljunemise tulemusena esinevad *P. infestans* Eesti populatsioonis pidevad fenotüübilised muutumised ja haigustekitaja mitmekesistumine (**I**, **II**). Sarnast tulemust kinnitavad ka genotüübilased uuringud, mis hiljuti Eesti populatsiooni kohta avaldati (Runno-Paurson *et al.*, 2016). Järelikult on eriti oluline patogeeni populatsioonide järjepidev jälgimine. Antud doktoritöö raames iseloomustati patogeeni Läti ja Leedu populatsioonide fenotüüpe ning võrreldi neid Eesti ja teiste Euroopa populatsioonidega.

Töö tulemustest selgus, et paarumistüüpide suhe *P. infestans*'i Eesti populatsioonis osutab sugulisele paljunemisele (**I**, **II**). Sarnaselt Eestiga esinesid ka Lätis ühtedel ja samadel põldudel A1 ja A2 paarumistüübid (**III**), paarumistüüpide (A1 ja A2) suhe Leedus oli kõikidel uuritud aastatel ligilähedane 1:1-le (**IV**). Neist tulemustest järeldub et haigustekitaja sugulise paljunemise tõenäosus nii Lätis kui Leedus on kõrge ja muldade saastumise oht kauapüsivate oospooridega on reaalne. Tulemustest selgus, et Balti riikides on patogeeni populatsioonid paarumistüüpide omavahelise suhte poolest sarnased ja kirjanduse andmetega võrreldes sarnanevad need Põhjamaade – Soome, Taani, Norra ning Rootsi populatsioonidega (Lehtinen *et al.*, 2008, Hannukkala, 2012), kuid samuti Poola (Chmielarz *et al.*, 2014) ning Loode-Venemaa (Statsyuk *et al.*, 2013) populatsioonidega. Saadud tulemustest võib järeldada, et lisaks Yueni ja Anderssoni (2013) väitele, kes leidsid et Põhjamaades esinevad *P. infestans* populatsioonid moodustavad Mehhiko kõrval teise sugulise paljunemise keskuse, lisanduvad sellesse keskusse ka Balti riikides esinevad populatsioonid.

Antud uuringutest selgus, et patogeeni metalaksüüli-resistentsus oli Eesti, Läti ja Leedu populatsioonides sarnane – domineerisid metalaksüülile tundlikud tüved (**II**, **III**, **IV**). Eesti populatsioonis on aastate jooksul toimunud suured muutused. Kui aastatel 2004–2005 (**I**) olid peaaegu pooled (49%) analüüsitud tüvedest metalaksüüliresistentsed, siis 2013 aasta analüüsist selgus, et on resistentsete isolaatide osakaal oli kahanenud 16%-ni ning tundlike tüvede osakaal tõusnud 64%-ni (**II**). Metalaksüülitundlikud tüved on viimasel kümnendil domineerinud ka Põhjamaades (Lehtinen *et al.*, 2008), Poolas (Chmielarz *et al.*, 2014), Venemaal Moskva regioonis (Statsyuk *et al.*, 2013), Valgevenes (Pobedinskaya *et al.*, 2011) ning Tšehhis (Mazáková *et al.*, 2011). Kõrge tundlike tüvede osakaal populatsioonis võib viidata harvaesinevale metalaksüüli kasutamisele analüüsitud põldudel. Väiketootjate jaoks on fungitsiidide kasutamine viimane abinõu, ees-

kätt just nende kalliduse tõttu. Samas viitab kõrge metalaksüüli-tundlike tüvede osakaal sellele, et metalaksüüli-põhised tõrjepreparaadid võivad haigustõrjes efektiivselt toimida.

Analüüs näitas, et kõik virulentsusfaktorid (R1–R11) esinesid kõigis Balti populatsioonides (**I**, **II**, **III**, **IV**) ning enim levinud virulentsusfaktorid olid R1, R2 (**II**, **IV**), R3, R4, R6 (**IV**), R7, R10, R11 (**I**, **II**, **III**, **IV**). Kõikides Balti riikide populatsioonides esinesid harva R5, R8 ja R9 virulentsusfaktorid (**I**, **II**, **III**, **IV**). Varasemaga võrreldes on Eesti viimastel aastatel R2 virulentsusfaktori osakaal kasvanud (**II**), selle esinemissagedus on samuti kõrge Läti ja Leedu populatsioonides (**III**).

Patogeeni rassiline struktuur oli väga varieeruv ja keeruline kõikides Balti riikides (**I**, **II**, **III**, **IV**). Ainulaadsete rasside osakaal Eesti populatsioonis oli 26%, Lätis 35% ning Leedus 36%. Kõigis uuritud populatsioonide proovide hulgas esines isolaate, mis olid kõikide resistentsusgeenide suhtes virulentsed, rassiga 1.2.3.4.5.6.7.8.9.10.11 (**I**, **II**, **III**, **IV**). Üks enim esinenud rass kõikides Balti riikides oli 1.2.3.4.6.7.10.11 (**I**, **III**, **IV**), välja arvatud 2013 aastal Eesti populatsioonis, kus domineerivaks rassiks oli 1.2.3.4.5.6.7.8.10.11 (**II**). Selle aasta tulemus erines teistest Euroopa populatsioonidest (Hermansen *et al.*, 2000; Lehtinen *et al.*, 2007; Lehtinen *et al.*, 2008; Runno-Paurson *et al.*, 2009, 2014). Keerulised rassid on Eestis tavalised (Runno-Paurson *et al.*, 2009, 2010, 2013, 2014), aga ka Venemaal (Statsyuk *et al.*, 2013) ning Poolas (Chmielarz *et al.*, 2014). Suur rassiline varieeruvus on tõenäoliselt seotud mõlema paarumistüübi (A1 ja A2) samaaegse esinemisega, mis loob eeldused suguliseks paljunemiseks ning sellele järgnevaks patogeeni populatsiooni mitmekesistumiseks.

Käesoleva uurimistöö tulemustest järeldub, et kolmes Balti riigis esineva patogeeni populatsioonid on fenotüübilt väga varieeruvad ning keerulised (**I**, **II**, **III**, **IV**). Patogeeni Eesti populatsioonides on viimasel kümnendil toimunud muutused metalaksüüli-resistentsuses, R2 virulentsusgeeni esinemissageduses, virulentsusrasside struktuuris ning Shannoni varieeruvusindeksis (**I**, **II**). Kui aastatel 2004–2005 (**I**) sarnanes Eestis esinev patogeen pigem Euroopas esinevaga (Hermansen *et al.*, 2000; Lehtinen *et al.*, 2008; Hannukkala, 2012; Runno-Paurson *et al.*, 2014), siis 2013 aastal (**II**) sarnanes see pigem Ida-Euroopas (Śliwka *et al.*, 2006; Statsyuk *et al.*, 2013) ja Põhjamaades levinud haigustekitajaga (Lehtinen *et al.*, 2008).

Tööst järeldub, et patogeenile toimis ajafaktor, kutsudes esile erinevusi patogeeni virulentsuses. Uuritavast regioonist ning kartulipõldude paiknemisest sõltusid paarumistüüpide proportsioonid, samuti leiti seosed haigustekitaja metalaksüüli-resistentsuse ning virulentsusfaktorite esinemise ning põldude asukoha vahel (**IV**). Viljelusviisist tingitud erinevusi *P. infestans* Eesti (**II**) ja Leedu (**IV**) populatsioonides ei leitud, küll aga mõjutas viljelusviis *P. infestans* Läti populatsioonis paarumistüüpide esinemissagedust (**III**).

Sugulise paljunemise tulemusena suudab *P. infestans* kohaneda erinevate ilmastikuoludega ning üle elada ebasoodsad keskkonnatingimused, mistõttu tuleb patogeeni arengut iga aasta järjepidevalt jälgida, et olla teadlik populatsioonis toimuvatest muutustest. Saadud teadmised on olulised tõrjemeetodite efektiivsemaks kohandamisel. Teave oospooride põhjustatud haiguspuhangute kvantitatiivse osakaalu kohta võib tõrjesüsteemide väljatöötamisel olulist lisandväärtust omada, mistõttu tuleks selle välja selgitamine lähitulevikus eesmärgiks võtta, kuna sellekohane uurimus on Balti riikides veel teostamata.

ACKNOWLEDGEMENTS

I am thankful to my grandmother and to my sister for believing in me, more than I have ever believed in myself. They both have been an inspiration and they have helped me to become the person I am today.

Last but not least, I am thankful to Risttee Congregation in Tartu and to all my brothers and sisters in Christ, who have inspired me to be a better person and who have opened their hearts to me and my family!

I

ORIGINAL PUBLICATIONS

The structure of *Phytophthora infestans* populations from organic and conventional crops

Eve Runno-Paurson · Triinu Remmel ·
Ann Ojarand · Alice Aav · Marika Mänd

Abstract The characteristics of populations of *Phytophthora infestans* from organic farms, small conventional farms and large conventional farms were determined from isolates collected in northern Estonia in 2004 and 2005. For the population as a whole 41% were A2; all virulence factors to the 11 R genes from *Solanum demissum* were found; and more than 70% had high or intermediate resistance to metalaxyl. Isolates from organic farms tended to have more complex pathotypes than isolates from either large or small conventional farms, but there was a higher proportion of metalaxyl resistant isolates from large conventional farms than from small conventional farms or from organic farms.

E. Runno-Paurson (✉) · A. Aav · M. Mänd
Institute of Agricultural and Environmental Sciences,
Estonian University of Life Sciences,
Kreutzwaldi 1,
Tartu 51014, Estonia
e-mail: eve.runno.paurson@emu.ee

T. Remmel
Department of Zoology, Institute of
Ecology and Earth Sciences, University of Tartu,
46 Vanemuise Street,
Tartu 51014, Estonia

A. Ojarand
Estonian Research Institute of Agriculture,
Department of Plant Biotechnology EVIKA,
Teaduse 6a, Saku,
75501 Harjumaa, Estonia

E. Runno-Paurson
Department of Biochemistry and Plant Protection,
Jõgeva Plant Breeding Institute,
Jõgeva alevik 48309, Estonia

Introduction

Potato late blight, caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases of potato worldwide. It is an ongoing threat to potato growers in temperate regions, requiring vigilance and often numerous applications of fungicide for effective control (Cooke et al. 2003). Under favourable conditions the pathogen not only reduces yield by destroying foliage and decreasing tuber growth, but also causes rotting of the tubers before and during storage (Smart and Fry 2001), thereby causing considerable further yield losses. In Estonia, it is not possible to achieve high yield with good quality in conventional potato production without using fungicides to control the late blight pathogen (Koppel 1997). In organic fields, where mostly varieties with high resistance are used, yield loss may reach 50% (Runno-Paurson *et al*, unpublished data). Copper based fungicides, which are used in organic production systems in Europe, are prohibited in Estonia.

Before the 1970s, European populations of the late blight pathogen appear to have consisted solely of a single clonal lineage of the A1 mating type, known as US-1, which has the mitochondrial DNA (mtDNA) haplotype Ib (Goodwin et al. 1994). In recent years, in most European populations, these 'old' population genotypes have not been detected. The 'new' genotypes comprise isolates of both mating types (Spielman et al. 1991; Day and Shattock 1997; Lebreton and Andrivon 1998); their coexistence allows the pathogen to reproduce sexually and to form oospores. Oospores can withstand unfavourable conditions and survive in the soil, thus affecting the epidemiology of the disease (Mayton et al. 2000). The new population also contains both Ia and IIa mtDNA haplotypes (Day and Shattock 1997; Lebreton and Andrivon 1998). In many cases, there has been an increase in the complexity of virulence phenotypes (Sujkowski et al. 1996; Hermansen et al. 2000; Lehtinen et al. 2008).

P. infestans reproduces sexually in most European countries (Zwankhuizen et al. 2000; Bagirova et al. 1998; Schöber-Butin 1999; Andersson et al. 1998; Brurberg et al. 1999; Lehtinen et al. 2007; Lehtinen et al. 2008; Avendaño Córcoles 2007; Śliwka et al. 2006). The proportion of A2 mating type isolates collected from commercial potato fields has remained low in France, Germany, Belgium and Switzerland (Gisi and Cohen 1996; Bakonyi et al. 2002a), whereas in the Netherlands, the Nordic countries and the UK it has exceeded 50% (Hermansen et al. 2000; Turkensteen et al. 2000; Lehtinen et al. 2007; Lehtinen et al. 2008, Lees et al. 2009).

In Estonia, the A2 mating type was first found in 1987. Data from 2002–2003 indicated the presence of both mating types at most study sites, suggesting the occurrence of sexual reproduction in Estonian populations (Runno-Paurson et al. 2009). In such a situation, management of the new sexually reproducing populations is a challenge for conventional production and can be crucial for the economy of organic potato producers (Hannukkala and Lehtinen 2005).

The number of organic farms in Estonia has increased since the early 1990s, notably since 2002. About 10 percent of all cultivated land is used for potato production. However, organic farms in Estonia are varied; for example, many of them do not rotate crops and the seed potatoes they use are often not certified. More importantly, with the prohibition of fungicide use, organic farms have a higher risk of late

blight epidemics and consequent yield loss than conventional fields.

The main objective of this study was to compare the population structure of *P. infestans* in organic and conventional productions in Estonia. It was postulated that *P. infestans* populations in organic production may differ in their resistance to fungicides or the diversity of certain phenotypic or genotypic traits (mating types, specific virulence, resistance to metalaxyl and mtDNA haplotypes) from those in conventional production. Higher diversity is likely to pose a higher risk of yield loss; for example, when two mating types co-occur and produce oospores, there is a higher risk of long-term survival of the pathogen. A higher diversity in virulence or fungicide resistance related traits can lead to a more effective selection of these traits. The results of this study can be compared with the populations in other regions of Estonia and other European countries to get a larger picture of the spatiotemporal variation in the population structure of this pathogen.

Materials and methods

Collection and isolation of isolates

In two consecutive years, 2004 and 2005, 196 isolates of *P. infestans* were collected from twelve potato fields (4 organic, 4 small scale conventional and 4 large scale conventional production) in northern Estonia (Table 1). The small and large scale conventional farms sampled differed in their use of agro-technical methods. In the small scale conventional farms, farmers used seed potatoes of uncertain quality and did not practise good crop rotation. Fungicides were applied only once per growing season. In the large scale conventional farms, farmers used high-quality certified potato seed, adhered to the recommended crop rotation, and made at least 6–7 treatments against potato late blight per season. Copper based fungicides are not used in Estonian organic production.

Nine to twenty-three leaflets, each with a single lesion (one per plant) were collected in organic and small scale farms twice in each year: at the beginning of the outbreak and at the end of the growing season (an approximately equal number of isolates was taken early and late in the season). In the early stages of the outbreak, approximately 20–25% of leaf area of the

Table 1 Sampling of *Phytophthora infestans* isolates collected from different cropping systems in Estonia (2004–2005)

Cropping system	Tested for		
	Mating type (n)	Virulence (n)	mtDNA haplotype (n)
Organic	42	54	24
Small scale conventional	61	68	24
Large scale conventional	72	74	18
Total	175	196	66

infected plants and less than 10% of plants were infected with late blight. In the later stages, about 20–40% of the leaf area and more than 50% of the plants were infected. On the large scale farms, samples were collected at the beginning of the outbreak. The plants were selected by randomising the distance from field edges, and from each plant the blighted leaf was also randomly chosen, excluding those that had several or no lesions.

Isolations were carried out by placing a fragment of infected leaf tissue between ethanol and flame-sterilized tuber slices. Tubers of susceptible cultivars without known R genes were used (Berber or Bintje). The slices were put into sterile Petri dishes with a moist filter paper disk on top. The Petri dishes were incubated for 6–7 days at 16°C in a growth chamber until the mycelia had grown through the slice. A small amount of mycelia from tuber slices was transferred with a sterile needle to rye B agar (Caten and Jinks 1968). The pure cultures were preserved at 5°C and transferred to rye agar after every 2 months. All phenotypic tests were carried out in October–November of the year of isolation. Mitochondrial DNA haplotype analyses were conducted in November–December 2005.

We used the data of field tests for foliage blight resistance (scores 1–9) from the EUCABLIGHT database (www.eucablight.org) to evaluate the late blight resistance of potato varieties grown in our study fields.

Phenotypic analyses

Mating types were determined by the method described in Runno-Paurson et al. (2009). Observed oospore formation in single isolate pure cultures was interpreted as the occurrence of self-fertility of the isolates. The tester isolates were the same as those described in Lehtinen et al. (2007). The resistance to metalaxyl of all 70 isolates was tested using a modification of the floating leaflet method (Hermansen et al. 2000) as described in Runno-Paurson et al. (2009). The specific virulence of each of the 196 isolates was determined using Black's differential set of potato genotypes containing resistance genes R1–R11 (Malcolmson and Black 1966) (provided by Scottish Agricultural Science Agency). Laboratory procedures were performed as described in Runno-Paurson et al. (2009).

Neutral marker assessment

At least four isolates were selected from each field (66 in total) for determination of the mitochondrial DNA (mtDNA) haplotype. The isolates were selected so that the proportion of mating types was approximately the same as in the main sample of the particular field. The mtDNA haplotype of the isolates in the subset was determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method of Griffith and Shaw (1998). The mtDNA haplotype detection was conducted at MTT Agrifood Research, Finland. Isolates were transplanted into 10 cm Petri dishes on pea agar and incubated until the surface of the agar was filled with mycelia. Then DNA was extracted and purified using Dneasy kit (Quiagen). The approximate DNA concentration in water solution was determined by comparing the fluorescence of the solution with that of standard solutions on agar plate containing ethidium bromide under UV light. A DNA concentration of 1 ng/μl or more in the PCR mix was required to get clear PCR product bands in the following electrophoresis. The DNA polymerase DyNAzyme II (Finnzymes) was used with a concentration of 0.02 U/μl in the PCR mix. The primers used for amplification of mt DNA regions P2 and P4 were F2 (5′-TTCCCTTTGTCCTCTACCGAT-3′) + R2 (5′-TTACGGCGGTTTAGCACATACA-3′) and F4 (5′-TGGTCATCCAGAGGTTTATGTT-3′)+R4 (5′-CCGATACCGATACCAGCACCAA-3′), 0.2 p mol/μl. The PCR program used for amplification of P2

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was 1x 90°C for 5 min, 35x (94°C for 30 s, 64°C for 1 min, 72°C for 1 min), 1x (72°C for 5 min, 11°C for 30 s and 4°C for ever). For P4, an annealing temperature of 55°C was used. Digestions with MspI (P2) and EcoRI (P4) were performed at 37°C overnight. The digested DNA samples were loaded into 1% agarose gel containing ethidium bromide. The restriction patterns were visualized using an UV transilluminator.

Data analysis

Statistical analyses were performed with the SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC, USA) using the GENMOD procedure. Logistic analyses were used to test for the dependence of mating type (multinomial response variable: A1, A2 or both) and haplotype (binomial: Ia vs. IIa) on locations (twelve fields) and years (2004 vs. 2005). Similar analyses were performed to compare the proportions of different mating types, haplotypes and isolates resistant to metalaxyl between cropping systems (small and large scale conventional fields and organic fields), i.e. all studied fields were assigned to one of these three groups.

Separate logistic analyses were used to test for the difference in the prevalence of virulence against different R genes (virulent vs. non-virulent) between years, the dependence of mating type on haplotype and race prevalence (unique vs. prevalent), and the association between virulence complexity (average number of R-genes overcome) and resistance to metalaxyl. The dependence of virulence complexity on cropping system was analysed with one-way ANOVA, as were the differences in the Shannon index values between cropping systems. Average susceptibility (scores 1–6, variety-specific scores were obtained from the Potato Late Blight Network For Europe website calculated from the results of foliage blight field tests) of potato plants grown in the different cropping systems to late blight was compared using a logistic model with an ordinal multinomial response variable. Race diversity

was calculated with the normalized Shannon diversity index (Sheldon 1969).

Results

Mating type determination

Among the 175 tested isolates, 57% were A1 mating type, 41% were A2 mating type and 2% were self-fertile. Both A1 and A2 mating types were detected from 11 of the 12 fields. The proportion of the A2 mating type in the isolates sampled in 2004 was lower than those sampled in 2005 (28% resp. 54%; $\chi^2=11.87$, d.f.=1, $p=0.0006$). There were further differences between cropping systems ($\chi^2=9.60$, d.f.=2, $p=0.0082$), the proportion of A2 being highest in organic fields and lowest in large scale conventional fields (Table 2).

Resistance to metalaxyl

In total, 110 isolates were screened for resistance to metalaxyl. In the 2 years, 49% of the isolates were resistant to metalaxyl, 34% were intermediate and 17% were classified as sensitive. Of the metalaxyl resistant strains, 65% were A1 mating type, 30% were A2 mating type and 5% were self-fertile; however, the association between metalaxyl resistance and mating type was not significant ($\chi^2=3$, d.f.=1, $p=0.083$).

Considerable differences between potato cropping systems were observed ($\chi^2=23.75$, d.f.=2, $p<0.0001$). In particular, in the large scale conventional fields, 66% of the tested isolates were resistant to metalaxyl, while in the small scale farm fields 26% and in the organic fields only 14% of the isolates were resistant (Table 3). There were no differences between years (2004 vs 2005, $\chi^2=0.98$, d.f.=1, $p=0.42$); however, when compared to the data collected in 2002–2003 (Runno-Paurson et al. 2009) the prevalence of metalaxyl resistant isolates had increased from 30 to 49% ($\chi^2=5.45$, d.f.=1, $p=0.02$).

Table 2 Percentages of mating types among isolates of *Phytophthora infestans* from different cropping systems in Estonia (2004–2005)

Cropping system	A1 (%)	A2 (%)	A1A2 (%)	Isolates tested (n)
Organic	38	62	0	42
Small scale conventional	61	39	0	61
Large scale conventional	65	31	4	72
Total	57	41	2	175

Table 3 Metalaxyl resistance among isolates of *Phytophthora infestans* from different cropping systems in Estonia (2004–2005)

Cropping system	Metalaxyl resistance[a]			
	R (%)	I (%)	S (%)	Total
Organic	14	52	33	21
Small scale conventional	26	42	32	19
Large scale conventional	66	26	9	70
Total	49	34	17	110

[a] S, metalaxyl-sensitive; I, intermediate metalaxyl-sensitive; R, metalaxyl-resistant

Virulence

All known virulence factors (to overcome genes R1–R11) were found among the 196 isolates. Nearly all isolates were virulent on differentials with genotypes R1, R3, R4, R7, R10 and R11. Virulence factor 9 (1%) was rare and factors 5 (10%) and 8 (10%) were relatively rare (Fig. 1, Table 4). A difference in the prevalence of virulence factors 2, 5, 8, and 9 was observed between the two sampling years (factor 2: $\chi^2=10.95$, d.f.=1, $p=0.0009$; factor 5: $\chi^2=9.38$, d.f.=1, $p=0.0022$; factor 8: $\chi^2=16.03$, d.f.=1, $p<0.0001$; factor 9: $\chi^2=5.55$, d.f.=1, $p=0.019$). After applying a Bonferroni correction (since eleven comparisons were made), only the differences in virulence factors 2, 5

and 8 remained significant. The three rarest virulence factors in Estonia, R5, R8 and R9, only appeared in the large scale conventional fields, while virulence factors R2 and R6 with relatively low frequencies were more prevalent in the organic fields than in the other cropping systems. Thirty-eight races were detected (Table 5). The two most common races made up 70% (Table 5) of the isolates tested. The overall virulence complexity (average number of R-genes overcome) was 6.7 (Table 5). Virulence complexity was highest in the organic farms (7.3). Complex races predominated in the organic fields, but were less common in the small and the large scale conventional fields ($F_{(193)}=8.49$, $p=0.00029$). The overall normalized Shannon diversity index was 0.38 and differed significantly between cropping systems ($F_{(2)}=23.89$, $p=0.0028$). This index was as high as 0.71 in the large scale conventional fields, but much lower in the small scale (0.13) and the organic fields (0.18).

Potato plants grown in large scale farms were on average less resistant to late blight than those grown in small scale and organic farms (Wald.stat=80.18, d.f.=2, $p<0.01$).

Mitochondrial DNA haplotype

Three mitochondrial haplotypes (Ia, IIa and IIb) were detected among the 66 isolates tested. Two isolates of haplotype IIb were found from the large scale

Fig. 1 Frequency (percentage) of virulence to potato R-genes among isolates of *Phytophthora infestans* collected from different cropping systems in Estonia (2004–2005)
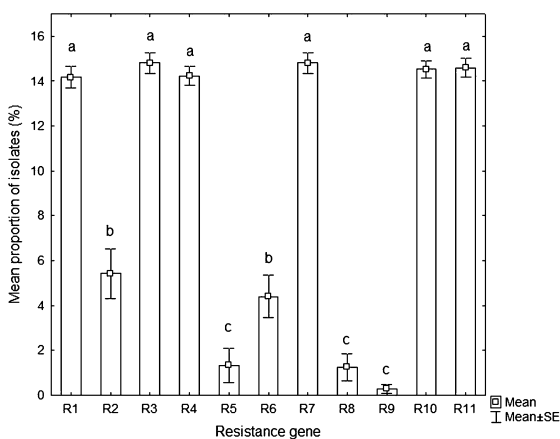
Table 4 Frequencies of specific compatibility (virulence) to potato R-genes in isolates of *Phytophthora infestans* from different cropping systems in Estonia (2004–2005)

Crop system	Virulence to resistance gene											Mean number of virulences/ isolate	Number of tested isolates
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11		
Organic	100	65	100	100	0	63	100	0	0	100	100	7.2	54
Small scale conventional	100	10	100	100	0	9	100	4	0	100	100	6.2	68
Large scale conventional	82	36	92	84	26	22	92	23	5	88	89	6.4	74
Total	95	38	98	95	5	31	98	4	1	97	97	6.6	196

conventional fields. The majority of isolates were haplotype IIa (74%) and the minority were Ia (23%). No significant differences were found in the frequencies of Ia and IIa between years ($\chi^2=1.46$, d.f.=1, $p=0.23$). However, differences between cropping systems were observed ($\chi^2=8.38$, d.f.=2, $p=0.015$), with the highest proportion of IIa in the large scale conventional fields and lowest in the organic fields. Interestingly, in the latter, only one haplotype (Ia) was detected (Table 6). There was no association between mating type and haplotype ($\chi^2=0.76$, d.f.=1, $p=0.38$). There were no differences between isolates taken early *vs* late during the season in the prevalence of different haplotypes; nor in the proportions of mating types, metalaxyl resistance or the number of R-genes overcome (statistics not shown).

Discussion

The results of this study suggest that there may be differences between potato cropping systems in various aspects of the population structure of *P. infestans* present in the fields. It is probable that different management practices, mainly fungicide use but also crop rotation and the source of potato seeds, are behind these differences. Dissimilarities were found in the prevalence of mating types, virulence genes, mtDNA haplotypes and resistance to metalaxyl. These results were also not always in accordance with those found in previous studies in this and other geographical regions, implying a noticeable spatial and temporal variation in *P. infestans* population parameters.

Nevertheless, the average proportion of A2 mating type found in this study (41%) was consistent with the

results of a previous study conducted in Estonia (Runno-Paurson et al. 2009; Runno-Paurson et al. 2010). Somewhat lower prevalence of the A2 mating type has been reported in Belgium (Bakonyi et al. 2002a), Finland (Hermansen et al. 2000; Lehtinen et al. 2007; Lehtinen et al. 2008) and Hungary (Nagy et al. 2006). Meanwhile, a higher proportion of A2 mating type has been detected in certain years in Austria (Avendaño Córcoles 2007), Czech Republic (Mazáková et al. 2006), Finland (Lehtinen et al. 2007), Denmark and Sweden (Lehtinen et al. 2008), Hungary (Bakonyi et al. 2002b), Poland (Śliwka et al. 2006) and the Netherlands (Zwankhuizen et al. 2000).

The presence of both mating types in the same field indicates the possibility of oospore production in potato foliage (Turkensteen et al. 2000). In this study, both mating types were detected at nearly all sites (92% of studied fields), with a single exception of an organic field in 2004. This percentage is based on just twelve fields, but it is supported by previous studies, conducted in 2002–2003 and 2004–2007, based on 32 and 28 fields, respectively (Runno-Paurson et al. 2009; Runno-Paurson et al. 2010), where the two mating types co-occurred in 88% of the fields. Similar frequencies of co-occurrence of the mating types have been reported from Germany (Bouws and Finckh 2007) where two mating types co-existed in 60–92% of the sites, and frequencies as high as 29–56% have been found in Nordic countries (Lehtinen et al. 2008). However, it is possible that the differences between studies arise from different numbers of isolates studied per field, rather than true differences in population composition, as the probability of detecting both mating types depends on sample size. This study did not support the previous findings that the co-occurrence of both mating types is more common

Table 5 Race frequencies among isolates of *Phytophthora infestans* from different crop productions in Estonia (2004–2005)

Crop system	Races	Number of virulence factors	Number of isolates
Organic	1.2.3.4.6.7.10.11	8	34
	1.2.3.4.7.10.11	7	1
	1.3.4.7.10.11	6	19
Small scale conventional	1.2.3.4.6.7.10.11	8	6
	1.3.4.7.8.10.11	7	3
	1.2.3.4.7.10.11	7	1
	1.3.4.7.10.11	6	58
Large scale conventional	1.2.3.4.5.6.7.8.9.10.11	11	3
	1.2.3.4.5.6.7.9.10.11	10	1
	1.2.3.4.5.6.7.8.10.11	10	1
	1.3.4.5.6.7.8.10.11	9	1
	1.2.3.4.6.7.8.10.11	9	1
	1.2.3.4.5.7.8.10.11	9	2
	1.2.3.4.5.6.7.10.11	9	1
	1.3.4.5.7.8.10.11	8	1
	1.3.4.5.6.7.10.11	8	1
	1.2.3.4.7.8.10.11	8	3
	1.2.3.4.6.7.10.11	8	1
	1.2.3.4.5.7.10.11	8	1
	1.2.3.4.5.7.10.11	8	3
	1.3.4.7.8.10.11	7	5
	1.3.4.6.7.10.11	7	2
	1.3.4.6.7.10.11	7	1
	1.3.4.5.7.10.11	7	2
	1.2.3.6.7.10.11	7	1
	1.2.3.4.7.10.11	7	1
	1.2.3.4.7.10.11	7	3
	1.3.4.7.10.11	6	14
	1.3.4.7.10.11	6	5
	2.4.7.10.11	5	1
	1.3.7.10.11	5	1
	1.3.4.7.11	5	1
	1.3.4.10.11	5	1
	3.4.7.10	4	1
	3.4.5.7	4	1
	2.3.4.7	4	1
	1.3.7.11	4	1
	7.10.11	3	1
	3.7.8	3	1
	3.7.10	3	1
	3.10.11	3	1
	1.7.11	3	1
	1.3.7	3	1
	6.10	2	1

Table 5 (continued)

Crop system	Races	Number of virulence factors	Number of isolates
	3.11	2	1
	3.10	2	1
	11	1	1
Total number of isolates			196
Total number of races			38

in organic fields, as has been reported from Finland (Lehtinen et al. 2007), southern Flevoland in the Netherlands (Zwankhuizen et al. 2000) and Scotland (Cooke et al. 2003). However, based on our results, differences in the A1/A2 ratio between cropping systems can be suggested, even though larger sample sizes are needed to explicitly prove this finding. For instance, in the organic fields, 62% of isolates were A2 mating type whereas in the large scale conventional farm fields only 31% of isolates were A2 mating type. The possibly higher prevalence of A2 mating type, both mating types found from most fields, and no rotation may presume higher risk for sexual reproduction in the organic fields than in the other cropping systems. Organic fields were also more severely infected than conventional crops, even though less susceptible potato varieties were used. The main reason for this is probably the lack of fungicide use in organic fields; however, an additional risk factor may be an increased oospore production, which reduces the effect of crop rotation if it is not performed sufficiently frequently (Lehtinen et al. 2007).

Further differences between cropping systems were evident in the resistance of isolates to metalaxyl fungicides. Metalaxyl resistant isolates were found four times more often in the large scale conventional fields than in the organic fields. This difference could be explained by the use of metalaxyl products in the large scale conventional fields, even though no significant differences were detected between the large scale conventional fields treated and not treated with metalaxyl (statistics not shown). Furthermore, it is possible that the overall prevalence of metalaxyl resistance has varied in recent years; in a previous study (Runno-Paurson et al. 2009), the average percentage of resistant isolates was 30%, but, in the present study was 49%. In both years, but especially in 2004, the epidemics started earlier and were more severe than those observed in 2002–2003 by Runno-Paurson et al. (2009). The intensive use of metalaxyl in Estonia against the heavy late blight pressure during those years may have contributed to this variation in resistance, although we cannot be certain that the difference is not coincidental.

The Estonian population of *P. infestans* is most similar in the frequency of virulence factors to those described recently in Nordic countries (Hermansen et al. 2000; Lehtinen et al. 2007, 2008), France and Switzerland (Lebreton and Andrivon 1998; Knapova and Gisi 2002; Pilet et al. 2005). The mean number of virulence factors found in Estonia (6.6) has remained at approximately the same level as in previous years (Runno-Paurson et al. 2009; Runno-Paurson et al. 2010); similar values were also found in Denmark (6.92) and Sweden (6.87) (Lehtinen et al. 2008) in 2003. Pathotype variability seems to have increased

Table 6 Number and percentages of mitochondrial DNA haplotypes among isolates of *Phytophthora infestans* from different cropping systems in Estonia (2004–2005)

Crop system	Number (and percentage) of isolates				
	Ia	IIa	Ib	IIb	Total
Organic	24 (100)	0 (0)	0 (0)	0 (0)	24
Small scale conventional	16 (67)	8 (33)	0 (0)	0 (0)	24
Large scale conventional	9 (50)	7(39)	0 (0)	2 (11)	18
Total	49 (74)	15 (23)	0 (0)	2 (3)	66

from the early 1990s in Norway and Finland from 5.3 and 5.8, respectively, (Hermansen et al. 2000) to 6.3 by 2002 (Lehtinen et al. 2007). A possible increase in the average number of virulence factors per isolate has also been noted in North-Western Russia since the late 1990s: from 6.3 to 7.7 in 2003 and 8.1 in 2007 (Vedenyapina et al. 2002; Zoteyeva and Patrikeeva 2008). However, the data are too few to rigorously confirm such an increase.

Race diversity calculated by the normalized Shannon diversity index showed a much lower value (0.38) in this study compared to the very high diversity among isolates collected from Estonia in 2002 to 2003 (0.89, Runno-Paurson et al. 2009). As a comparison, in a sample of 432 isolates collected in 2004–2007, pathogen diversity was still relatively low (0.54) (Runno-Paurson et al. 2010). Interestingly, even though lower values of diversity have been found in newer studies, the average virulence complexity was relatively high. The diversity index was much higher among isolates collected from large scale conventional fields. This result is particularly surprising because, unlike smaller farms, the large scale farms used certified potato seed tubers and practiced rotation. The reason for this may lie with the seed source used in those farms. Large scale farms grow potato varieties imported directly from western Europe, mostly from the Netherlands, where the local populations have highly complex virulence spectra (8–10 virulence factors per isolate) and the proportion of A2 is extremely high (Van Raaij et al. 2008). Large quantities of seed potato are also imported from Germany and Denmark. The mean number of virulences per tested isolate was found to be 6.9 in Denmark and 6.2 in Germany, and the frequency of A2 mating type was over 50% in Denmark and 13–46% in Germany, with both mating types co-existing in 76% of the fields, on average (Bouws and Finckh 2007; Lehtinen et al. 2008). It is therefore likely that the higher diversity of the *P. infestans* populations in large scale farms is caused by mixing local genotypes with strains imported from other, highly diverse populations.

Moreover, compared to small conventional and organic farms, the potato varieties used in large conventional farms were on average more susceptible to late blight, which may have contributed to the high pathogen diversity. A high variability of pathogens does not always pose a higher threat to the hosts, even though it can potentially promote the pathogen

population to adapt more quickly with new host plant varieties, or to express virulence against a higher number of resistance genes. Nevertheless, a single pathotype can cause severe damage, as in the case of Great Britain where 80% of the population consists of only one aggressive genotype 13_A2, determined by SSRs and mating type (Lees et al. 2009). It can also be noted that, even though pathogen diversity tended to be higher in large conventional fields in our study, the plants were still more affected in organic fields.

Another dissimilarity found between cropping systems was the higher prevalence of the generally less common IIa haplotype in the large conventional fields compared to the other field types. This may also be explained by a larger number of imported pathogens in those fields. The high proportion of Ia haplotype (74%) in this study differs from the results of the previous study conducted in Estonia (46%, Runno-Paurson et al. 2009). A higher proportion of Ia haplotype has also been observed in Poland, England, Scotland, Wales, the Netherlands and France (Lebreton and Andrivon 1998; Cooke et al. 2003; Lebecka et al. 2007). Haplotype IIb was found for the first time in Estonia. The Ib haplotype, associated with the old clonal *P. infestans* populations present in Europe during most of the 20th century (Spielman et al. 1991), was not found.

The markers used were chosen to show mainly phenotypic variability, with genetic variation characterized by mtDNA haplotypes. The occurrence of A1 mating type isolates with three different mtDNA fingerprints clearly indicates that there is some genotypic diversity in the population. In a previous study (Runno-Paurson et al. 2010), the Shannon index of genotypic diversity, obtained by DNA fingerprinting with probe RG57, was particularly high in large conventional fields. In addition, the high numbers of rare genotypes detected every year indicate that oospores may act as an infection source in organic and conventional potato fields (Zwankhuizen et al. 2000). In further studies it would also be informative to use microsatellite markers to detect the specific relationships between phenotypic and genotypic variation, as reported in Guo et al. (2009).

In conclusion, the results of this study clearly suggest that there may be cropping system-specific differences in the population structure of *P. infestans*, which most probably arise from different management practices in these systems. Such differences can likely lead to variation in the risk of yield loss. In contrast to the previous assumptions, several aspects

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of pathogen diversity, such as genotypic diversity, race complexity and the diversity of mtDNA haplotypes appeared to be highest in the large conventional fields. On the other hand, the proportion of the novel A2 mating type and virulence complexity were highest in the organic fields. The prevalence of metalaxyl resistance was also highest in the large conventional fields. Such differences should not be ignored by producers, and different precautions can be suggested for managing different types of farms. In particular, conventional farmers may benefit from the use of other control methods beside metalaxyl fungicides to limit the spread of resistance in the pathogen population. The spatiotemporal variation observed in *P. infestans* population parameters across Europe may imply that managers also need to consider the regional situation to make optimal decisions. However, it would certainly be desirable to repeat these comparisons in further studies incorporating a larger number of fields to confirm more rigorously the differences between management practices. Importantly, the separate effects of crop rotation, chemical control, seed source and host resistance need to be addressed.

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Runno-Paurson, E., Kiiker, R., **Aav, A.**, Hansen, M., Williams, I. H. 2016.

DISTRIBUTION OF MATING TYPES, METALAXYL SENSITIVITY AND VIRULENCE RACES OF *PHYTOPHTHORA INFESTANS* IN ESTONIA.

Agronomy Research,14 (1), 20–2

Distribution of mating types, metalaxyl sensitivity and virulence races of *Phytophthora infestans* in Estonia

E. Runno-Paurson[1*], R. Kiiker[1], A. Aav[1], M. Hansen[1] and I. H. Williams[1]

[1]Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Department of Plant Protection, Kreutzwaldi 1, Tartu 51014, Estonia;
*Correspondence: eve.runno-paurson@emu.ee

Abstract. Potato late blight, caused by the oomycete *Phytophthora infestans*, is a destructive potato disease, causing considerable crop loss worldwide. As the late blight pathogen population is diverse and variable in Estonia, changes in the population should be monitored regularly. In this study, the Estonian population of *P. infestans* was characterised with mating type, sensitivity to metalaxyl and virulence on potato R-gene differentials. During the growing season 2013, 110 isolates were collected from nine potato fields. The frequency of A2 mating type was on average 29%, and varied significantly between different fields from 7% to 78% (*p*=0.001). On all studied potato fields, both mating types were recorded, suggesting continuous sexual reproduction of *P. infestans* and possible risk of oospore production and early attacks of late blight in Estonian potato fields. The prevalence of metalaxyl sensitive isolates in the population (64%) differed from results from previous research. Thus changes have occurred in the *P. infestans* Estonian population. There were no significant differences in metalaxyl sensitivity between studied fields (*p*=0.073). The Estonian race structure was highly diverse and complex, on average 7.2 virulence factors per isolate, but varied between fields from 5.6 to 9.0. 42 virulence races were found; the four most common were 1.2.3.4.5.6.7.8.10.11, 1.2.3.4.6.7.8.10.11, 1.2.3.4.7.10.11 and 1.2.3.4.7.8.10.11, which comprised 46% of the population. The overall normalized Shannon's diversity index was 0.69, confirming the high diversity of the population. Continuous pathogen population studies describing the contemporary *P. infestans* population are essential in order to advise potato breeders and growers accordingly.

Key words: mating type, metalaxyl, *Phytophthora infestans*, population variation, potato late blight, virulence testing.

INTRODUCTION

Potato late blight is caused by the oomycete pathogen *Phytophthora infestans*; it first appeared in Europe more than 160 years ago but remains a major threat to potato crops both in Europe and worldwide. Despite recent active research and progress, late blight still requires vigilance and often numerous applications of fungicide for effective control (Cooke et al., 2011). It is a serious problem for Estonian potato production, particularly under favourable conditions, when it can destroy the whole potato haulm and the average loss due to late blight can reach 20–25% and in untreated fields even more (Runno-Paurson et al., 2010). Fungicides are used routinely in conventional potato production, but under favourable conditions for the disease, with heavy pressure from the pathogen, protection of large areas is complicated (Runno-Paurson et al., 2010).

Potato late blight pathogen *P. infestans* is an heterothallic organism with two mating types A1 and A2. The pathogen is able to reproduce sexually and asexually. During the 1980s, *P. infestans* populations containing both mating types migrated from Mexico apparently in 1976/77 into Europe giving rise to sexually reproducing populations (Fry & Goodwin, 1997). New diverse *P. infestans* genotypes were very adaptable and spread quickly all over Europe displacing the old clonal lineage which is now found only rarely (Carlisle et al., 2002; Cooke et al., 2011). *P. infestans* benefits from sexual reproduction by increased adaptability of the pathogen and production of oospores that can survive in the soil for several years (Turkensteen et al., 2000; Yuen & Andersson, 2013). While low temperature could even conserve the viability of the oospores (Turkensteen et al., 2000; Hannukkala, 2012) the pathogen benefits from it in Estonia during cold winters. Furthermore, genotyping *P. infestans* Estonian isolates with SSR markers revealed high genetic diversity and provided evidence that sexual reproduction and recombination are common in this population (Runno-Paurson et al., 2016).

While the late blight pathogen population is diverse and variable in Estonia, changes in the population should be monitored regularly. Therefore, in this study, potato late blight pathogen *Phytophthora infestans* isolates collected in 2013 from different potato fields in Estonia were characterised with phenotypic characteristics such as mating type, metalaxyl sensitivity and virulence to Black's differentials.

MATERIALS AND METHODS

Collection and isolation of *P. infestans* strains

Potato leaves infected by *P. infestan*s were collected in 2013 from nine sites from five counties in Estonia (Table 1). The samples were taken from large scale conventional and small scale conventional growers' potato fields, potato field trials and an organic field (Table 1). In large scale conventional productions farmers used high-quality certified seed potatoes and applied fungicide 6–8 times per season. The metalaxyl-based fungicide Ridomil Gold MZ 68 WG was applied at the beginning of disease infection at least twice. Rotation varied between 0–2 years on these farms. Small scale conventional farmers used uncertified seed potatoes and no fungicides routinely for late blight control. The interval between growing potato crops was 1–2 years. At the potato field trials in Reola and at the Estonian Crop Research Institute in Jõgeva fungicide was not used, but at Lepiku field trial fungicides were applied three times per season. At the organic field and field trials growers rotated fields and grew potatoes every 3–4 years.

Nine to fifteen isolates were cultured from each sampling site (Table 1). The sampled plants were located at a random distance from field edges. Only single lesion leaves were collected from each plant, taken randomly; any leaves with several or no lesions were excluded. Isolations were carried out and maintained using methods described by Runno-Paurson et al. (2009). All phenotypic tests were carried out immediately after the isolations were finished (October to January). *P. infestans* isolates of this study are preserved at Tartu Fungal Collection (TFC).

Table 1. The number of *Phytophthora infestans* isolates collected in 2013 and tested for mating type, metalaxyl sensitivity and virulence phenotype

Sampling site	County	Crop type*	Mating type (n)	Metalaxyl (n)	Virulence (n)
			Tested for		
Jõgeva 1	Jõgeva	Trial field	12	12	12
Jõgeva 2	Jõgeva	Organic	14	14	14
Antsla	Võru	LSC	14	14	13
Lepiku	Tartu	Trial field	15	15	13
Reola	Tartu	Trial field	11	11	11
Sürgavere	Viljandi	LSC	9	9	8
Tilga	Tartu	SSC	11	11	11
Verioramõisa	Põlva	LSC	14	14	14
Võnnu	Tartu	SSC	10	10	8
Total			110	110	104

* - LSC (large scale conventional), SSC (small scale conventional)

Phenotypic analyses

Mating types were determined by the method described in Runno-Paurson et al. (2009). The tester isolates were 90209 (A1) and 88055 (A2) as described in Hermansen et al. (2000). Isolates forming oospores on plates with the A1 mating type were registered as A2; isolates that formed oospores with the A2 mating type were registered as A1.

P. infestans isolates resistance to metalaxyl was tested using a modification of the floating leaflet method (Hermansen et al., 2000). Leaf disks (14 mm diameter) were cut with a cork borer from leaves of five-week-old greenhouse-grown potato plants. The susceptible cultivar 'Berber' was used. Six leaf disks were floated abaxial side up in Petri plates (50 mm diameter) each containing 7 mL distilled water or metalaxyl in concentrations of 10.0 or 100.0 mg L-1 prepared from technical grade metalaxyl-M (Syngenta experimental compound (metalaxyl-M), CGA 329351A). The inoculation and trial incubation was done as described by Runno-Paurson et al. (2009). The isolates were rated resistant if they sporulated on leaf disks in 100 mg L^{-1} metalaxyl (Hermansen et al., 2000). Those sporulating on leaf disks in a metalaxyl concentration of 10 mg L^{-1}, but not on leaves floating on 100 mg L^{-1} were rated intermediate, and those sporulating only in water were rated sensitive.

The virulence pathotype was determined with detached leaflet set of Black's differentials of potato genotypes containing resistance genes R1–R11 from *Solanum demissum* (Malcolmson & Black, 1966) (provided by the Scottish Agricultural Science Agency). Laboratory procedures were as described in Runno-Paurson et al. (2009).

Data analysis

Statistical analyses were performed with the SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC, USA). Differences in the prevalence of the two mating types of *P. infestans* isolates between study sites were tested using a logistic analysis (GENMOD

procedure in SAS) with a multinomial response variable (A1, A2, or both). Analogous logistic procedures were used to examine the differences in the resistance to metalaxyl (a multinomial response variable: resistant, intermediate or sensitive) between sites and also between different mating types. The dependence of specific virulence (percent of isolates that show virulence against particular R-genes) on site and R-genes was analysed with type III ANOVA and Tukey HSD post-hoc tests (α=0.05). In all analyses, "site" was treated as a categorical variable.

Race diversity was calculated with the normalized Shannon diversity index (Sheldon, 1969). The differences in the Shannon index values between sites was analysed with one-way ANOVA and Tukey HSD test.

RESULTS AND DISCUSSION

Both A1 and A2 mating types were found among *P. infestans* isolates collected in Estonia in 2013. Out of 110 tested isolates, 71% were classified as A1 mating type and 29% were classified as A2 mating type (Table 2). There were considerable differences in the proportion of A1 and A2 between sampling sites (*Chi-square* = 25.44, *df* = 8, *p* = 0.001), with the frequency of A2 mating type varying between different potato fields from 7 to 78% (Table 2).

Table 2. Percentages of mating types among isolates of *Phytophthora infestans* in Estonia in 2013

Site	Mating type (%)		Number of isolates
	A1	A2	
Jõgeva 1	75	25	12
Jõgeva 2	71	29	14
Antsla	43	57	14
Lepiku	93	7	15
Reola	91	9	11
Sürgavere	22	78	9
Tilga	73	27	11
Verioramõisa	93	7	14
Võnnu	60	40	10
Total	71 ± 7.6*	29 ± 7.6*	110

* – mean ± SE

The average percentage of A2 mating type was a bit lower than previously recorded in Estonia (Runno-Paurson et al., 2010; 2013; 2014), but temporal fluctuation has been noticed before (Runno-Paurson et al., 2012). However, our findings on mating types in the Estonian population are generally comparable with populations described recently in studies from the Nordic countries Finland, Denmark, Norway and Sweden (Lehtinen et al., 2008; Hannukkala, 2012), Latvia (Aav et al., 2015), Lithuania (Runno-Paurson et al., 2015), Poland (Chmielarz et al., 2014) and the north-western part of Russia (Statsyuk et al., 2013). In this study both mating types were recorded from all studied potato fields

(Table 2), indicating continuous sexual reproduction of *P. infestans* and possible risk of oospore production and early attacks of late blight in Estonian potato fields. As a result of sexual reproduction high genetic diversity in the population can also be expected as shown in the previous *P. infestans* collection from 2004 characterized with SSR markers (Runno-Paurson et al., 2016).

Of the 110 isolates tested for metalaxyl response, 64% were sensitive, 20% were intermediate and 16% were resistant to metalaxyl (Table 3). Significant differences were not found between sampling sites (*Chi-square* = 24.83, *df* = 16, *p* = 0.07).

The prevalence of metalaxyl sensitive isolates in the population showed clearly different results compared to previous research done in Estonia (Runno-Paurson et al., 2010; 2014). This suggests changes in the *P. infestans* Estonian population. These results are similar to recent findings from Latvia (Aav et al., 2015), Lithuania (Runno-Paurson et al., 2015), Poland (Chmielarz et al., 2014) and other European populations of *P. infestans*. The significant association between response to metalaxyl and mating type was not found (*Chi-square* = 1.11, *df* = 2, *p* = 0.57).

Table 3. Metalaxyl sensitivity among isolates of *Phytophthora infestans* from Estonia in 2013

	Percentage of isolates			Number of isolates
	S*	I*	R*	
Jõgeva 1	75	8	17	12
Jõgeva 2	57	7	36	14
Antsla	64	36	0	14
Lepiku	67	20	13	15
Reola	100	0	0	11
Sürgavere	78	11	11	9
Tilga	55	36	9	11
Verioramõisa	36	36	28	14
Võnnu	50	20	30	10
Total	64 ± 5.8**	20 ± 4.4**	16 ± 4.1**	110

* – S, metalaxyl sensitive; I, intermediate metalaxyl sensitive; R, metalaxyl resistant

** – mean ± SE

All 11 known virulence factors were found among the 104 *P. infestans* isolates tested for virulence (Fig. 1). Almost all isolates were virulent on differentials with genotypes R1, R2, R3, R4, R7, R10 and R11. Virulence factors 9 (11%) and 5 (28%) were relatively rare (Fig. 1). No significant differences in virulence factors were found between field sites ($F_{(8,80)}$ = 2.64, *p* = 0.11). The Estonian race structure was highly complex, on average 7.2 virulence factors per isolate, but varied between fields from 5.6 to 9.0. The overall normalized Shannon's diversity index was 0.69 and varied between potato fields from 0.43 to 0.95 (Table 4), confirming the high diversity of the population. 42 virulence races were found and the four most common virulence races were 1.2.3.4.5.6.7.8.10.11, 1.2.3.4.6.7.8.10.11, 1.2.3.4.7.10.11 and 1.2.3.4.7.8.10.11,

comprising 46% of the population (Table 5). Twenty-six races were unique and found only once (Table 5).
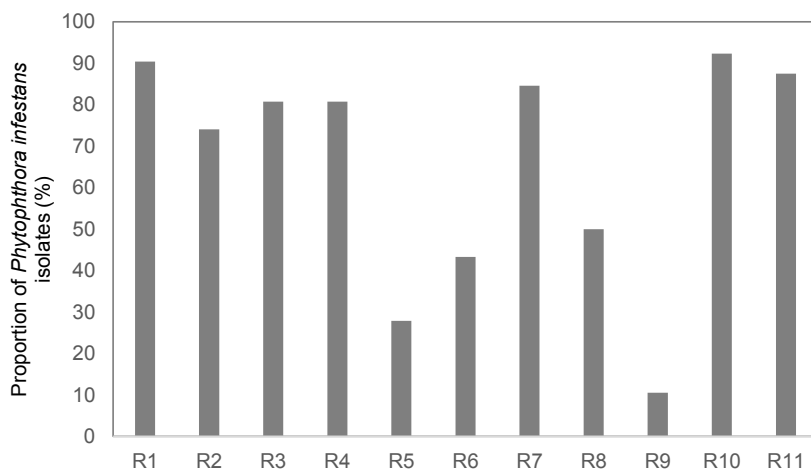

Figure 1. Frequency of virulence to potato R-genes in the Estonian population of *Phytophthora infestans* in 2013.

The frequencies of virulence factors in this study were similar to those reported recently in Estonia (Runno-Paurson et al., 2009; 2010; 2014), except for virulence factor 2, the frequency of which has increased over the years. The prevailing race 1.3.4.7.10.11 of *P. infestans* in most European populations (Hermansen et al., 2000; Lehtinen et al., 2008; Hannukkala, 2012; Chmielarz et al., 2014; Runno-Paurson et al., 2014) was found only four times (3.8%) from the Estonian population. The average number of virulence factors per isolate was 7.2, which is quite similar to that found in other populations from Estonia in previous long-term studies (Runno-Paurson et al., 2012; 2014) and also among Eastern European populations (Śliwka et al., 2006; Statsyuk et al., 2013; Aav et al., 2015; Runno-Paurson et al., 2015).

Table 4. Racial diversity of *Phytophthora infestans* from different sites in Estonia in 2013

Site	Hs*
Jõgeva 1	0.43
Jõgeva 2	0.87
Antsla	0.75
Lepiku	0.72
Reola	0.58
Sürgavere	0.75
Tilga	0.95
Verioramõisa	0.80
Võnnu	0.92
Total	0.69

* – the normalized Shannon diversity index for race diversity calculation

Table 5. Race frequencies among isolates of *Phytophthora infestans* from Estonia in 2013

Races	Number of virulence factors	Number of isolates
1.2.3.4.5.6.7.8.10.11	10	16
1.2.3.4.5.6.7.9.10.11	10	3
1.2.3.4.5.6.7.10.11	10	3
1.2.3.4.6.7.8.10.11	9	11
1.2.3.4.6.7.9.10.11	9	3
1.2.3.4.5.7.8.10.11	9	2
1.2.3.4.7.8.10.11	8	10
1.2.3.4.6.7.10.11	8	3
1.2.3.4.5.7.10.11	8	2
1.2.3.4.7.10.11	7	11
1.3.4.7.8.10.11	7	2
1.3.4.7.10.11	6	4
1.4.7.10.11	5	2
1.7.10	3	2
1.10	2	2
10	1	2
Races found once		26
Total number of isolates		104
Total number of races		42

CONCLUSIONS

In this study both mating types of *P. infestans* were recorded in all studied potato fields, indicating continuous sexual reproduction and possible risk of oospore initiated early attacks of late blight in these fields. As metalaxyl-sensitive isolates dominated in the pathogen population sensible and moderate use of metalaxyl based fungicides in most fields could be employed. Overall, the *P. infestans* population in Estonia is highly diverse and complex, characterised by high virulence race diversity. Continuous pathogen population studies describing the contemporary *P. infestans* population are essential in order to advise potato breeders and growers accordingly.

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III

Aav, A., Skrabule, I., Bimšteine, G., Kaart, T., Williams, I. H., Runno-Paurson, E. 2015.

THE STRUCTURE OF MATING TYPE, METALAXYL RESISTANCE AND VIRULENCE OF *PHYTOPHTHORA INFESTANS* ISOLATES COLLECTED FROM LATVIA.

The structure of mating type, metalaxyl resistance and virulence of
***Phytophthora infestans* isolates collected from Latvia**

Alice AAV[1], Ilze SKRABULE[2], Gunita BIMŠTEINE[3], Tanel KAART[4],
Ingrid H. WILLIAMS[1], Eve RUNNO-PAURSON[1]

[1]Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences
Kreutzwaldi 1, Tartu, 51014, Estonia
E-mail: alice.aav@emu.ee

[2]Priekuli Plant Breeding Institute
Zinatnes 2, Priekuli, LV4126, Latvia

[3]Institute of Soil and Plant Sciences, Latvia University of Agriculture
Lielâ iela 2, Jelgava, LV-3001, Latvia

[4]Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences
Kreutzwaldi 1, Tartu, 51004, Estonia

Abstract

Potato late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most serious potato (*Solanum tuberosum* L.) diseases, causing considerable yield loss in potato production worldwide, including Latvia. At present, the data on the population characteristics of *P. infestans* in Latvia is sparse. Therefore, the main aim of this study was to collect new data on Latvian isolates of *P. infestans*, to determine the main characteristics of the pathogen, particularly mating types, metalaxyl resistance and virulence with Black's differential set of potato genotypes containing resistance (R) genes R1–R11.

During 2010–2012, 181 isolates of *P. infestans* were collected from 23 potato fields, from 13 locations in Latvia. Out of 181 isolates tested, 52.5% were A1, 43.1% – A2 and 4.4% – self-fertile mating type. Of 116 isolates screened for resistance to metalaxyl, 25.9% were resistant, 19.8% – intermediate and 54.3% – sensitive. More than 80% of isolates were virulent to R1, R3, R4, R7, R10 and R11, while 33% or fewer isolates were virulent to R5, R8 and R9. The least frequent was virulence against R9 in 24% of isolates. Our study revealed that the Latvian population of *P. infestans* is diverse. The proportion of mating types and the occurrence of both A1 and A2 in the same field indicate the possibility of sexual recombination in Latvian fields. Thus, it is very important to keep the crop rotation system, to prevent soil contamination with long-living oospores. The Latvian population of *P. infestans* shares many similarities with other European populations, which suggests gene flow between populations.

Key words: mating type, metalaxyl resistance, potato late-blight, virulence.

Introduction

Potato late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most serious potato (*Solanum tuberosum* L.) diseases in the world, which has been studied for a long time by different plant pathologists worldwide. The disease can cause severe potato famine, as happened in Ireland in 1845, resulting in the displacement/death of 1/3 of Irish people (Fry, 2008). Despite all the research on *P. infestans*, it remains a major problem in agriculture and one of the worst crop diseases in the world. Control of potato late blight can be achieved by using anti-oomycete fungicides regularly (Cooke et al., 2012), but this is expensive, for example, in Europe, it costs one billion euros (including

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fungicides and unusable crops) (Haverkort et al., 2008), and the canopy must be treated weekly, when inoculum is already present, and the weather conditions are favourable for disease spread (Lehtinen et al., 2007). In Latvia, potato late-blight is a serious problem for both small-garden and large scale potato growers, who have to apply fungicide to protect plants from infection. For instance, in the vegetation period with favourable weather conditions for late blight development, tuber yield formation was interrupted two weeks earlier in an organic potato production field without any fungicide compared to in a conventional field where fungicide was applied (Skrabule, 2010). Late blight development depends on potato varietal resistance, the foliage of potato varieties with higher resistance were less damaged by late blight, as was also found in Denmark, Estonia, Poland, Lithuania and Latvia (Hansen et al., 2005). Thus, it is very important to use only certified seed material of resistant potato varieties and to keep a proper crop rotation (Bimšteine, 2008) thereby reducing spending on fungicides and contamination of the environment and food.

 P. infestans is heterothallic and can be either A1 or A2 mating type (Drenth et al., 1995). Both mating types can reproduce asexually which is a very fast way for the disease to spread. Yuen and Andersson (2013) reported that, for example, in North and South America, Asia and Africa, *P. infestans* reproduces mainly asexually. For sexual reproduction it has to produce gametangia and needs both A1 and A2 mating types to be present resulting in the production of oospores, which can survive in the soil for up to ten years without a susceptible host (Drenth et al., 1995). Until the early 1980s, the A1 mating type dominated European populations of *P. infestans* (Gisi, Cohen, 1996). The first report in Europe, on mating type A2, was from Switzerland in 1981 (Spielman et al., 1991) but since then, the A2 mating type has been found in almost every European country (Gisi, Cohen, 1996). The "old" population was displaced by the "new" and more aggressive population which resulted in earlier outbreaks of the disease (Fry et al., 1993).

 Recent studies in Great Britain have discovered a new aggressive lineage of *P. infestans*, which can overcome host resistance (Cooke et al., 2012). The pathogen has adapted well in different conditions thus securing its survival, for example in Northern Europe (also in Toluca Valley, Mexico), it has become a soil borne pathogen due to its ability to reproduce sexually (Yuen, Andersson, 2013) and does not need tubers or plant debris to survive over winter. At the same time Kildea et al. (2013) have reported finding *P. infestans* strains, similar to the former dominant population which had disappeared for more than thirty years (except one finding in 1990s), in Great Britain. Populations of *P. infestans* thus undergo continuous change and scientists face the challenge to outrace the pathogen development.

 At present, there is insufficient data on population characteristics of *P. infestans* in Latvia. The identification of *P. infestans* races during 1974–1990 was carried out by the Institute of Phytopathology of Soviet Union (Russia), and the A2 mating type of *P. infestans* was recorded for the first time in Latvia in 1987 (Bebre et al., 2004). Earlier studies have reported that formation of oospores takes place in Latvian potato fields, but so far, the ratio between mating types A1 and A2, is unknown (Bimšteine, 2008).

 Potato is a very important food source in Latvia. Potatoes are used mainly for marketing and also for the food industry producing potato chips and starch. Those of lower quality are sometimes used for animal feed (Cudere, 2008). Most farms (97%) produce potatoes on less than 1 ha and only 0.5% of farms grow potatoes on more than 5 ha. The average number of treatments against late blight in farms with a plantation size up to 0.5 ha is 1 and in farms with a larger potato area, 3–5 times (Cudere, 2008). The average yield losses in Latvian agroecological conditons are 15–30%, but in favourable years for late blight development, potato yield losses reach 30–60% (I. Skrabule unpublished data).

 Potato late blight has considerable importance in Latvian potato production. Therefore in this study, *P. infestans* isolates collected from Latvia were tested to determine the main characteristics of the late blight pathogen like mating types, metalaxyl resistance and virulence, in order to help develop and optimize control strategies against late blight, and to share this information with scientists (potato breeders), advisors and potato growers. It is also very important to compare populations of *P. infestans* in Latvia with other European populations of the pathogen.

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Materials and methods

Collection and isolation of isolates of Phytophthora infestans. During 2010–2012, 181 isolates of *P. infestans* were collected from different regions and different agro-intensity farms (23 potato fields, from 13 locations) in Latvia (Fig. 1, Table). The late blight samples were collected from large scale conventional farm fields (12), small scale conventional farm fields (6), organic fields (3) and trial fields with breeding lines (2).

Figure 1. Map of Baltic States and surrounding countries showing the regions in Latvia from which the isolates of *Phytophthora infestans* were collected during 2010–2012

All of the isolates were collected from infected leaves, each with a single lesion. All the collected leaflets were placed between washed and sliced potato tubers and transported in cool conditions to the laboratory. Collection of the isolates took place at the beginning of the outbreak of late blight until 97% of the foliage was destroyed. To culture the pathogen, tubers of the susceptible cultivar 'Berber', without known R-genes were used. Tubers were sterilised in ethanol, flamed and then sliced. Infected leaf tissues were placed between tuber slices, put into sterile Petri dishes and incubated in a growth chamber for 6–7 days at 16°C. When the mycelia of *P. infestans* had grown through the slice, a small amount of it was taken with a sterile needle, and placed in a sterile Petri dish, filled with rye B agar (Caten, Jinks, 1968). Phenotypic tests were carried out after culturing the pathogen.

Mating type determination. Mating type was determined for 181 isolates (Table) by growing each sample isolate together with the appropriate tester strain (A1 and A2), kindly sent by Asko O. Hannukkala (Natural Resources Institute Finland, Luke), in a Petri dish on rye agar. Plates were assessed after the mycelia had grown together, approximately 14 days after placing the Petri dishes into a growth chamber at 16°C. Determination was carried out under a microscope. Isolates forming oospores on plates with the A1 mating type but not the A2 were registered as A2; isolates that formed oospores with the A2 mating type but not the A1 were registered as A1; isolates that formed oospores with the A1 and the A2 were registered as self-fertile.

Table. Sampling of *Phytophthora infestans* isolates collected from different sites in Latvia (2010–2012)

Location	Year	Number of fields	Number of isolates	Number of isolates tested for		
				mating type	metalaxyl resistance	virulence
Cirulis	2010	1	3	3	3	1
Dipeni	2010	1	5	5	2	3
Igali	2010	1	5	5	2	2

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Priekuli	2010	1	13	13	7	4
Silmaci	2010	1	3	3	0	–
Vanagi	2010	1	7	7	5	2
Dobele	2011	2	3	3	1	3
Bauska	2011	2	4	4	2	4
Riga	2011	1	1	1	0	1
Priekuli	2011	3	35	35	22	35
Paukulite	2011	1	8	8	6	8
Vijciems	2011	1	7	7	3	6
Bilska	2011	1	8	8	6	8
Vijciems	2012	1	4	4	4	4
Bilska	2012	1	8	8	8	8
Priekuli	2012	3	53	53	35	53
Dreimani	2012	1	14	14	10	14
Total		23	181	181	116	156

Resistance to metalaxyl. Resistance to metalaxyl was tested for 116 isolates (Table), using a modification of the floating-leaflet method (Hermansen et al., 2000). The susceptible cultivar 'Berber' was used for the test. Potato plants were approximately five-six weeks old, when the leaves were taken and disks (15 mm diameter) cut from them with a cork borer. Six leaf disks were floated in Petri plates (50 mm), each containing 7 ml distilled water or water and metalaxyl solution with concentrations 0.0, 10.0 and 100.0 mg l^{-1}. The pathogen's sporangia were taken from Petri dishes, where they were grown in pure cultures on rye B agar, and put into distilled water with a pallet. All the leaf disks were infected with a suspension of sporangia (20 µl drop). After inoculation, Petri dishes were maintained in the growth chamber at 16°C and 90% relative humidity. The assessment was carried out after seven days by the same parameters as described in Runno-Paurson et al. (2009). The isolates were registered as resistant if they sporulated on leaflets in 100 mg l^{-1} metalaxyl, intermediate if they sporulated in a metalaxyl concentration of 10 mg l^{-1} but not on leaves floating on 100 mg l^{-1}, sensitive if they sporulated only in water (0 mg l^{-1} concentration metalaxyl).

Virulence tests. The specific virulence of 156 isolates (Table) was determined using Black's differential set of potato genotypes containing resistance (R) genes R1–R11 (Malcolmson, Black, 1966) provided by the Scottish Agricultural Science Agency, United Kingdom. Due to technical and logistic reasons, virulence tests were carried out in 2010 only for some of the isolates. Potato leaves were obtained from the differentials grown from meristem plants in the greenhouse. The meristem plants were reproduced by Plant Biotechnological Research Centre EVIKA in Saku, Estonia. Leaves were cut from the plants at 6–8 weeks of age. Filter paper was put in the bottom of plastic trays and moistened with distilled water. Leaves were placed lower side up on the filter paper and each leaflet was inoculated with a 20 µl drop of *P. infestans* sporangial suspension ($1.0–4.0 \times 10^4$ sporangia ml^{-1}). The trial was carried out in four replications. The plastic trays were covered with polyethylene after the inoculation to maintain high relative humidity and were incubated at 16°C with a 16 h light period. The results were assessed after seven days, using the same scale as indicated for the assessment of metalaxyl resistance.

Data analysis. To test for the effects of year (2010, 2011 or 2012), field type (organic, breeding, small scale conventional or large scale conventional) and their interaction on metalaxyl resistance (metalaxyl-sensitive, intermediate metalaxyl-sensitive or metalaxyl-resistant) and mating type (A1, A2 or self-fertile) multinomial models considering also random effect of site nested to field type were fitted with the *SAS 9.4* procedure *GLIMMIX*. As no effects were statistically significant irrespective of considering or not random site effect and the smallest *p*-values corresponded to year and field type interaction indicating potential presence of more specific relationships, we continued with less general studies applying the Fisher exact test to find the associations of metalaxyl resistance and mating type with sampling year by field types and with field types by sampling year. The Fisher exact test was applied also to study the relationships between metalaxyl resistance and mating type.

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The race diversity was calculated with the normalized Shannon diversity index. The pairwise comparison of virulence frequencies against different potato R-genes was performed with logistic model (*SAS 9.4* procedure *GLIMMIX*) considering simultaneously effects of year, field type, their interactions with R-genes and random effect of site nested to field type and followed by the Tukey test for multiple comparison of least square means. Associations between virulence to single potato R-gene and metalaxyl resistance, mating type, year and field type were tested for with the Fisher exact test. Each series of p-values concerning 11 potato R-genes was corrected for multiple testing using the Holm method.

To discover common patterns in virulence against R-genes hierarchical cluster analysis and principal component analysis were performed. The last was used also to study the relationships of sampling year, field type, mating type and metalaxyl resistance with common virulence patterns. Fisher exact tests and all multivariate analyses were performed with statistical software *R 3.1.1*, for principal component analysis package *ade4* was applied. Test results with $p < 0.05$ were considered statistically significant.

Results

Mating type. Of the 181 isolates tested, 52.5% were A1, 43.1% – A2 and 4.4% – self-fertile mating type. The distribution of mating types did not differ between years ($p = 0.55$) or between field types ($p = 0.40$) (Fig. 2). The interaction effect of year and field type was on the limit but still statistically non-significant ($p = 0.060$).
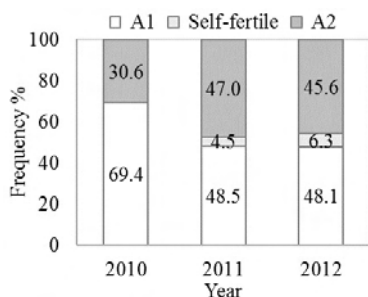

Figure 2. Frequency of mating types in Latvia (2010–2012)

However, omitting the year 2010, where no breeding and organic fields were presented, a statistically significant association between mating type and field type was found ($p = 0.004$) – if A1 mating type had almost the same frequency in all type of fields, then A2 mating type was found mostly in large scale conventional fields (56.7% of findings). Comparison of years 2011 and 2012 revealed that this association between mating type and field type persisted in general but with some changes. In 2011, the A1 mating type was found most frequently in large scale conventional fields and less frequently in organic fields (40.6% and 9.4% of findings, respectively), whereas in 2012, the opposite occurred with 31.5% of findings from organic fields and only 15.8% from large scale conventional fields. The A2 mating type was most frequently found in large scale conventional fields in both years, but the frequencies differed quite a lot (45.2% and 66.7% in 2011 and 2012, respectively). In 2011, 29.0% of A2 mating types were from breeding fields, but in 2012, only 5.6% of A2 findings came from such fields. The self-fertile mating type was found only in small and large scale conventional fields.

Metalaxyl resistance. Of the 116 isolates screened for resistance to metalaxyl, 25.9% were resistant, 19.8% intermediate and 54.3% sensitive to metalaxyl. Although these proportions varied with year, the association between metalaxyl resistance and year was not statistically significant ($p = 0.21$) (Fig. 3).
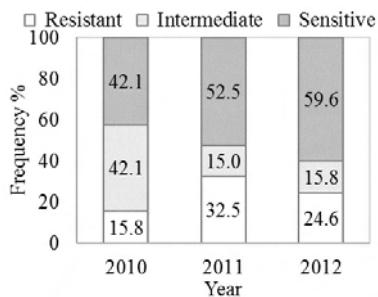
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Figure 3. Metalaxyl resistance among isolates of *Phytophthora infestans* in Latvia (2010–2012)

Metalaxyl resistant strains were absent from seven fields, five of which were large scale conventional fields, one a small scale conventional and one an organic field. Sensitive strains were absent from four fields, from one organic field and three large scale conventional fields. The distribution of metalaxyl resistance was quite similar over different field types ($p = 0.66$) and this tendency did not depend on year ($p = 0.13$).

The percentage of sensitive isolates was the highest among mating type A1 and the percentage of resistant isolates among mating type A2 (Fig. 4). However, this association was not statistically significant ($p = 0.093$). But leaving out the self-fertile mating type with only three isolates screened for resistance to metalaxyl resulted in significant association between mating type and metalaxyl resistance ($p = 0.044$). Studying the association by years revealed that the statistically significant association was found only in 2012 ($p = 0.006$ and $p = 0.003$, respectively considering and not the self-fertile mating type). Studying the distribution of metalaxyl resistance over years by mating types revealed a statistically significant change for mating type A1 ($p = 0.014$) but not for mating type A2 ($p = 0.56$).
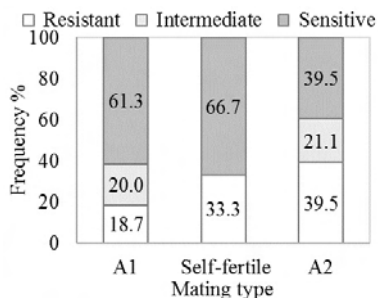

Figure 4. Metalaxyl resistance among isolates with different mating type in Latvia (2010–2012)

Virulence. Of the 156 isolates analysed for virulence, 4 (2.6%) were not virulent against any R-genes and 12 (7.7%) were virulent against all 11 R-genes. More than 80% of isolates were virulent to R1, R3, R4, R7, R10 and R11, while 33% or less isolates were virulent to R5, R8 and R9. The least frequent was virulence against R9 with 24% of isolates. In total 69 different pathotypes were detected and 94.1% of isolates were virulent against four or more R-genes. At the same time, the four most common pathotypes made up 33.6% of isolates and no pathotypes exceeded 10% of isolates. The most common races were 1.2.3.4.7.10.11 (15 isolates) and 1.2.3.4.6.7.10.11 (13 isolates). The normalized Shannon diversity index was 0.73.
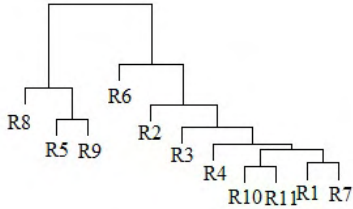
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Figure 6. Dendrogram of potato R-genes according to hierarchical clustering

The first two principal components describe 44.7% of total variability of virulence against different R-genes. The most common pattern (the first principal component) distinguishes less and more virulent isolates (Fig. 7) and the second pattern distinguishes isolates virulent against less and more common R-genes.
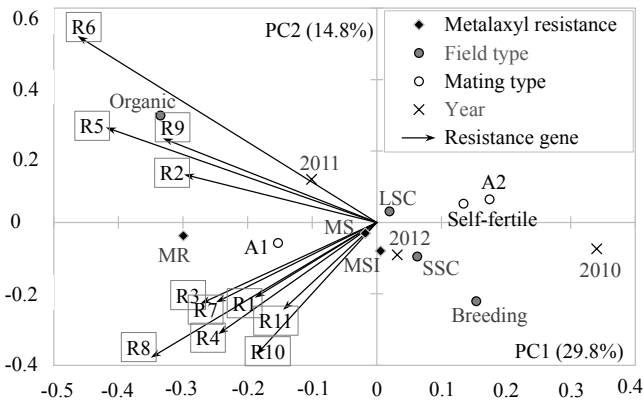
Note. Arrows correspond to factor loadings relating the first two principal components with R-genes and markers denote the average scores corresponding to different values of metalaxyl resistance (MS – metalaxyl-sensitive, MSI – intermediate metalaxyl-sensitive, MR – metalaxyl-resistant), field type (SSC – small scale conventional, LSC – large scale conventional), mating type (self-fertile) and year (2010–2012).

Figure 7. Results of principal component analysis of R-genes

The analysis of principal components' values (scores) shows that the virulence was generally more frequent in organic fields and among A1 and metalaxyl-resistant isolates and less frequent in 2010. The virulence against more rare R-genes appeared more frequently in organic fields and in 2011 and less frequently in breeding fields and among intermediate metalaxyl-sensitive isolates.

Discussion

P. infestans isolates, collected from different regions and different agro-intensity farms in Latvia, were tested to determine the main characteristics of the late blight pathogen and to compare populations of *P. infestans* in Latvia with other European populations of the pathogen. In our study, we found that the mating type A1:A2 ratio in 2011 and 2012 was close to 1:1, which is perfect for sexual reproduction and formation of oospores to take place (Turkensteen et al., 2000). This can be a direct threat to the strategies

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used to prevent late blight disease and to fight against the pathogen, because it becomes a soil-borne inoculum (Yuen, Andersson, 2013). The percentage of A1 mating type was higher than the incidence of A2 mating type for all three years. This result is similar to data from Estonia in 2001–2007 (Runno-Paurson et al., 2012) and from Lithuania in 2010–2011 (Runno-Paurson et al., 2015). In 2004–2007, the proportion (A1:A2:self-fertile) in Estonia was 64:33:3 (Runno-Paurson et al., 2010), which is close to the results in Latvia in 2010. The lower proportion of A2 mating type in 1998–2002 has also been reported in Ireland (Cooke et al., 2006), but, in 2009 and 2010, the numbers were totally different – nearly 60% and 75%, respectively, were A2 mating type (Cooke et al., 2012). In our study, A2 mating type was found mostly in large scale conventional fields – the direct opposite result to Estonian studies, where the proportion of A2 mating type was significantly higher in small scale conventional fields (Runno-Paurson et al., 2010). The spreading of A2 mating type poses a threat to sustainable potato production, because it is able to overcome previously late blight resistant varieties (Cooke et al., 2012). The fact that both A1 and A2 mating types are present in most of the studied Latvian fields can lead to soil contamination with oospores so it is very important to maintain the crop rotation system, to prevent early outbreaks of the disease. All this data supports the evidence that the northern population of *P. infestans* in Europe along with Baltic countries Latvia and Estonia (Runno-Paurson et al., 2014) can be called the second centre of sexual reproduction of *P. infestans* (Yuen, Andersson, 2013). The first finding of oospores in Latvia took place in the 1980s, but the research was discontinued. After that, the occurrence of oospores on potato leaves in Latvia was investigated during 2002–2004. In 80–94% of the investigated cases, oospores were found. Most of the oospores were formed on the potato plants in private gardens, where potatoes had been cultivated for many years, and the lowest frequency of oospores was in a certified organic field, where over twenty years other crops had been grown (Bimšteine, 2008). However, there was no information about the ratio of the two mating types.

Our results showed, that isolates sensitive to metalaxyl, were predominant. The results were similar to those obtained in the Nordic countries – Norway, Sweden, Finland and Denmark (Lehtinen et al., 2008) and Poland, where 58.3% of isolates were sensitive in 2006 (Chmielarz et al., 2014), and in 2007–2009, the proportion of metalaxyl-sensitive strains reached 75%. Isolates sensitive to metalaxyl dominated also in Lithuania in 2010-2012 (Runno-Paurson et al., 2015) and in 2011 as well as 2013, over 50% of tested isolates were sensitive to metalaxyl in Northern Ireland (<eucablight.org>). Nevertheless, opposite results have been reported in Ireland either (in 2009), where metalaxyl-resistant isolates dominated both populations – in the Republic of Ireland and in the Northern Ireland, and in France, where the frequency of metalaxyl-resistant strains reached 75% (<eucablight.org>). More stable results were reported in Estonia during 2004–2007, where the proportion of resistant-intermediate-sensitive strains was 37:25:37 percentages, respectively, but still, in 2007, metalaxyl-sensitive strains dominated the population. Another resemblance with the Estonian study is the fact that most of the metalaxyl-resistant isolates were A2 mating type (Runno-Paurson et al., 2010). The amount of metalaxyl-resistant strains of *P. infestans* in Latvia may indicate low usage of fungicides on the fields from which the samples were collected. For example, in Finland, the proportion of metalaxyl-based products is less than 10% of all fungicides used to prevent late blight (Hannukkala et al., 2007) and at the same time, in Estonia, metalaxyl-based fungicides are widely used (Runno-Paurson et al., 2014). In Denmark, metalaxyl-M is not registered for commercial use at all (Lehtinen et al., 2008).

In this study, we found 69 different pathotypes among the 156 isolates tested. In total 34.6% of the races were unique and found only from one isolate. The result is similar to Estonian studies in 2002–2003, where nearly half of the pathotypes were found only once (Runno-Paurson et al., 2009). The mean number of virulence factors per isolate was as high as 7.2. Twelve isolates were virulent to all R-gene differentials (1.2.3.4.5.6.7.8.9.10.11). In Finland (1997–2000), only one similar isolate was found, and, at the same time, the most common race in Finland and in Estonia (2002–2003) 1.3.4.7.8.10.11 (Lehtinen et al., 2007; Runno-Paurson et al., 2009), was not found in Latvia. The most common races were 1.2.3.4.7.10.11 (15 strains) and 1.2.3.4.6.7.10.11 (13 strains), whereas the dominating race in Europe and in Nordic countries 1.3.4.7.10.11 was found only five times. Complex races are also common in Polish populations (Chmielarz et al., 2014), a similar situation to that in Russia (Statsyuk et al., 2013) and in Estonia (Runno-Paurson et al., 2009; 2010; 2013; 2014). Frequency of virulence against R2 has been rare

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in European countries like Poland, Denmark, Finland, Norway and Sweden (Lehtinen et al., 2008; Chmielarz et al., 2014), but it is relatively high in Latvia, where 76% of the isolates were able to overcome the R-gene. Virulence factors R5 (27%), R8 (32%) and R9 (24%) were rare, but the incidence of those R-genes was still comparatively high when compared with results reported in Estonian populations in 2001–2007, where only 9% of the isolates were virulent to R5 and 11% to R9 (Runno-Paurson et al., 2013). To compare our results with research done in the 1960s and 1970s, there have been major changes considering *P. infestans* races in Latvian populations – predominant races 1, 4 and 1.4 were replaced in less than twenty years with more complex races 4.10.11, 1.4.7.8.10.11, 1.3.4.7.8.10.11 and 1.2.3.4.7.8.10.11 (Bebre et al., 2004), as in most European populations. Yuen and Andersson (2013) reported that using specific resistance (R) genes, as a preventative method against *P. infestans*, can be useless due to the pathogen's evolutionary potential to break through the resistance created with these genes, but this study showed that there are still a few (R5, R8 and R9) R-genes that may be useful if used in parallel with other protective methods.

Our study indicated that the Latvian population of *P. infestans* is diverse. The proportion of mating types and the occurrence of both A1 and A2 in the same field indicate the possibility of sexual recombination in Latvian fields. Due to this, it is very important to keep the crop rotation system, to prevent soil contamination with long-living oospores. The Latvian population of *P. infestans* shares many similarities with other European populations suggesting gene flow between populations.

Conclusions

1. The Latvian population of *Phytophthora infestans* is diverse. The proportion of mating types and the occurrence of both A1 and A2 in the same field indicate the possibility of sexual recombination in Latvian fields; therefore, it is essential to keep the crop rotation system to prevent soil contamination with oospores and early outbreaks of the disease in the fields.

2. Isolates sensitive to metalaxyl were predominant but were still absent from four (one organic and three large scale conventional) fields. About one fourth of the studied strains were resistant to metalaxyl.

3. A little more than one third of races found were unique and the mean number of virulences per isolate was 7.2. Twelve isolates were able to overcome all 11 (1.2.3.4.5.6.7.8.9.10.11) resistance (R) genes and the dominating race in Europe and in Nordic countries 1.3.4.7.10.11 was found only five times.

4. Latvian population of *P. infestans* shares many similarities with other European populations, which suggests gene flow between populations.

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Phytophthora infestans izoliatų, surinktų Latvijoje, dauginimosi tipo struktūra, atsparumas metalaksilui ir virulentiškumas

A. Aav[1], I. Skrabule[2], G. Bimšteine[3], T. Kaart[4], I. H. Williams[1], E. Runno-Paurson[1]

[1]Estijos gyvybės mokslų universiteto Žemės ūkio ir aplinkos mokslų institutas
[2]Priekulės augalų selekcijos institutas, Latvija
[3]Latvijos žemės ūkio universiteto Dirvožemio ir augalininkystės mokslų institutas
[4]Estijos gyvybės mokslų universiteto Veterinarinės medicinos ir gyvulininkystės mokslų institutas

Santrauka

Bulvių maras, sukeltas oomicetų *Phytophthora infestans* (Mont.) de Bary, yra viena grėsmingiausių bulvių ligų, dėl kurios patiriami dideli derliaus nuostoliai visame pasaulyje, taip pat ir Latvijoje. Šiuo metu yra nedaug duomenų apie *P. infestans* populiacijos ypatybes Latvijoje. Todėl pagrindinis tyrimo tikslas – surinkti naujų duomenų apie Latvijos *P. infestans* izoliatus, taip pat naudojant Black'o diferencialų rinkinį, kurį sudaro R1–R11 atsparumo genų, nustatyti svarbiausias šio patogeno savybes, ypač dauginimosi tipą, atsparumą metalaksilui ir virulentiškumą.

2010–2012 m. laikotarpiu iš 13 Latvijos vietovių bei 23 bulvių laukų buvo surinktas 181 *P. infestans* izoliatas. Iš 181 tirtų izoliatų 52,5 % buvo A1, 43,1 % – A2 ir 4,4 % – nelytinio dauginimosi tipo. Iš 116 izoliatų, tirtų dėl atsparumo metalaxilui, 25,9 % buvo atsparūs, 19,8 % –vidutiniškai atsparūs ir 54,3 % – jautrūs. Daugiau kaip 80 % izoliatų buvo virulentški R1, R3, R4, R7, R10 ir R11, o 33 % ar mažiau izoliatų – R5, R8 ir R9 genams. Rečiausias virulentiškumas R9 genui buvo nustatytas 24 % izoliatų. Tyrimas atskleidė, kad Latvijos *P. infestans* populiacija yra nevienoda. Dauginimosi tipų proporcija ir A1 bei A2 tipų pasiskirstymas tame pačiame lauke rodo lytinės rekombinacijos galimybę Latvijos laukuose. Todėl, siekiant užkirsti kelią užsikrėtimui ilgaamžėmis oosporomis, yra labai svarbu laikytis sėjomainos. Latvijos *P. infestans* populiacija turi daug panašumų su kitomis Europoje pasitaikančiomis populiacijomis, o tai rodo genų plitimą tarp populiacijų.

Reikšminiai žodžiai: atsparumas metalaksilui, bulvių maras, dauginimosi tipas, virulentiškumas.

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Runno-Paurson, E., Ronis, A., Hansen, M., **Aav, A.**, Williams, I. H. 2015.

LITHUANIAN POPULATIONS OF *PHYTOPHTHORA INFESTANS* REVEALED HIGH PHENOTYPIC DIVERSITY.

Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz - Journal of Plant Diseases and Protection, 122 (2), 57–65.

Lithuanian populations of *Phytophthora infestans* revealed a high phenotypic diversity

Eve Runno-Paurson[1,*], Antanas Ronis[2], Merili Hansen[1], Alice Aav[1] & Ingrid H Williams[1]

[1] Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, Tartu 51014, Estonia

[2] Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry Instituto ave. 1, Akademija, Kėdainiai distr., Lithuania

* Corresponding author: eve.runno-paurson@emu.ee

Abstract

This is the first characterisation of Lithuanian populations of *Phytophthora infestans* with mating type and virulence on a differential set of 11 R genes of *Solanum demissum*. The sensitivity to metalaxyl was also determined. In 2010–2012, 93 *Phytophthora infestans* isolates, collected from all over Lithuania, showed quite high and stable frequency of A2 mating type. On 45% of all studied potato fields, both mating types were recorded, suggesting sexual reproduction of *P. infestans* and possible oospore production in Lithuanian potato fields. Fourteen metalaxyl-resistant isolates were found among 71 isolates in the current study period, and sensitive isolates prevailed in all three years. Amongst the studied 70 isolates, 38 avirulence pathotypes were found. The Lithuanian race structure was highly diverse and complex (the average number of missing avirulence factors per isolate was 7.2). Most pathotypes were unique, appearing only once, and the four most common pathotypes comprised only 34% of the population.

Key words: potato late blight, phenotypic characterisation, diversity

Introduction

Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is one of the most important diseases of potato worldwide. In the Nordic region during the last decade, there are indications of earlier outbreaks of late blight, requiring more frequent fungicide treatments per season to control the disease (Hannukkala 2012). Long-term observation data from Finland and Estonia show that the first findings of blight now occur one month earlier than 20 years ago and blight outbreaks are more severe (Hannukkala 2012, Runno-Paurson et al. 2013a).

In Lithuania, potato late blight is a serious problem for potato production when weather conditions are favourable, with average yield losses varying between 5 to 50 percent (Ronis & Tamošiūnas 2005). Control of the disease is complicated as quite a large proportion of this area is still traditionally managed. Most (90%) potatoes are grown by small-scale growers (potato production on less than 5 ha) on family farms; most are grown with no or very low chemical protection, with spraying too late, resulting in considerable yield reduction (Ronis & Tamošiūnas 2005). In contrast, large scale potato producers in Lithuania apply fungicides 4–6 times per season (Valskytė 2000, Ronis & Tamošiūnas 2005). Currently, more than 16 different products are available for potato late blight control on the Lithuanian market. For conventional farmers, the most widespread active ingredients are mancozeb combined with dimethomorph, metalaxyl-M, propamocarb-HCl + fluopicolide, cyazofamid, or fluazinam whereas copper is registered for use on organic farms.

The late blight control problem is made more serious since mostly susceptible or quite susceptible potato cultivars are grown (A. Ronis unpublished data). Similarly the late blight resistance evaluation survey showed that most of western European potato cultivars are too susceptible to be grown without chemical protection under North-East European conditions (Runno-Paurson et al. 2013a). In Lithuania, the cultivar 'Aista' is partially resistant to late blight, but it is not preferred by growers because of its lateness, high starch content (up to 21%) and most importantly poor taste value (Asakavičiūtė et al. 2009). Similarly, potato cultivars with greater resistance are not preferred for large-scale production in northern and western Europe (Cooke et al. 2011, Runno-Paurson et al. 2013a).

The fungal-like oomycete *P. infestans* is heterothallic with two mating types, A1 and A2, enabling the pathogen to reproduce both sexually and asexually (Fry & Goodwin 1997). Before 1980, the worldwide population (except in Mexico) of the late blight pathogen appeared to be asexual and to consist of a single clonal lineage of A1 mating type. After the migration of new strains of *P. infestans* to Europe in the late 1970s and the introduction of the second mating type A2 apparently by potato imports from Mexico (Fry & Goodwin 1997), sexual reproduction of the pathogen became possible. The new genotypes spread very quickly and displaced the old clonal lineage in Europe which latterly has been found only rarely (Cooke et al. 2011). Sexual reproduction results in oospores which can overwinter in the soil (Hannukkala 2012, Yuen & Andersson 2013). The presence of sexual reproduction changes the epidemiology of this important potato disease, and thus changes the way in which disease control must be approached (Yuen & Andersson 2013). The effects of this change in the population have been clearly noted in potato fields, where epidemics have started earlier and the number of fungicide treatments needed for control of the blight have increased, probably partly because of oospore-derived infections (Hannukkala 2012). Also, there are indications of an increase in the complexity of virulence phenotypes since the 2000 s (Hannukkala 2012, Statsyuk et al. 2013).

Nevertheless, the population structure in some European countries such as Great Britain, France, Switzerland, Netherlands, Belgium, Denmark is clonal with a few dominating genotypes and sexual reproduction is rare (Gisi et al. 2011, Cooke et al. 2012, Li et al. 2012). Recent studies from northern and northeastern European populations have been characterized by a frequency and ratio of both mating types enabling sexual reproduction and the phenotypic variability is very high among the populations of *P. infestans* from these regions (Lehtinen et al. 2007, 2008; Runno-Paurson et al. 2009, 2010, 2011, 2012, 2013b, 2014; Hannukkala 2012, Statsyuk et al. 2013). These studies are strongly supported by Sjöholm et al. (2013) whose results show clearly that the late blight pathogen *P. infestans* reproduces sexually in the Nordic countries. The available evidence from northern Europe strongly supports regular sexual reproduction of *P. infestans* in this region (Yuen & Andersson 2013).

Little is known about Lithuanian populations of *P. infestans*, with the exception of a metalaxyl resistance study during 1990−96 at the Lithuanian Institute of Agriculture (Valskytė 2000). Valskytė started to investigate metalaxyl resistance problems in 1989 when resistance problems first appeared in Lithuanian potato fields. However, there is no knowledge about the A2 mating type and phenotypic genotypes within *P. infestans* populations in Lithuania, a gap in the European population map for this disease.

Therefore, the main objectives of the present study were to determine the general characteristics of the Lithuanian population of *P. infestans* in terms of mating type, reaction to metalaxyl and phenotypic diversity. More specifically we were interested to find out: 1) does the mating type ratio in Lithuania suggest occurrence of sexual reproduction? 2) what is the phenotypic variation within the Lithuanian population of *P. infestans* indicated by mating type, metalaxyl resistance and virulence and how does it compare with other European populations? In addition, the impacts of time, region and the influence of agricultural management practices were studied.

Materials and methods

Collection and isolation of isolates

Potato leaves infected by *P. infestans* were collected from 22 sites (five sites in 2010, 11 sites in 2011 and six sites in 2012) within the main potato growing areas (4 regions) of Lithuania (Fig. 1, Table 1). The potato fields from which the samples were taken were classified as small or large-scale conventional production fields or experimental field plots. The conventional producers were divided into two groups due to differences in their agrotechnical management practices. Small-scale conventional farmers, who are in the majority in Lithuania, use seed potatoes of poor quality and do not use fungicides routinely for late blight control. The first preventive fungicide applications are usually made too late for disease epidemic control; applications may vary from none to four. Large areas of potato are thus unprotected by late blight fungicides. In large-scale conventional production, farmers use high-quality certified seed potatoes and apply
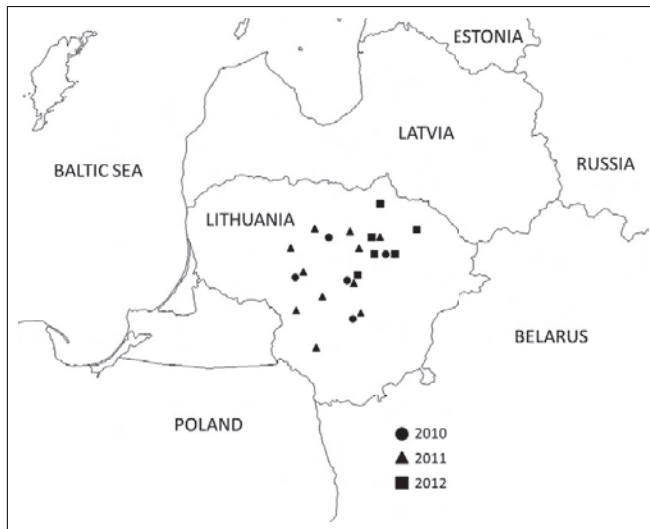

Fig. 1: Field sites in Lithuania from where late blight samples were collected during 2010−12.

Table 1: Time, origin, crop type, cultivar, state epidemic level and numbers of collected *Phytophthora infestans* isolates in Lithuania during 2010–2012

Sampling date	Location	Crop type	Cultivar	State of epidemic level	Number of isolates
07.08.2010	Akademija	experimental plots	'Laura'	beginning	6
07.08.2010	Raguvėlė	small-scale	unknown	beginning	3
07.08.2010	Tautušiai	small-scale	unknown	beginning	5
07.08.2010	Urniežiai	large-scale	'Laura'	beginning	5
07.08.2010	Šeduva	small-scale	unknown	beginning	2
16.08.2011	Daugėlaičiai	small-scale	unknown	middle	8
16.08.2011	Kaulakiai	small-scale	unknown	end	2
16.08.2011	Krekenava	large-scale	unknown	beginning	1
16.08.2011	Naisiai	small-scale	unknown	middle	2
16.08.2011	Panevėžys	large-scale	'Laura'	beginning	8
16.08.2011	Tytuvėnai	small-scale	unknown	end	7
17.08.2011	Akademija	experimental plots	'Laura'	middle	4
17.08.2011	Ariogala	small-scale	unknown	end	3
17.08.2011	Marijampolė	small-scale	unknown	middle	2
17.08.2011	Šakiai	small-scale	unknown	end	2
17.08.2011	Urniežiai	large-scale	'Laura'	middle	3
09.08.2012	Akademija	experimental plots	'Faxe'	beginning	5
09.08.2012	Kavarskas	small-scale	unknown	middle	3
09.08.2012	Latavenai	small-scale	unknown	middle	1
09.08.2012	Paežeriai	small-scale	unknown	middle	14
09.08.2012	Raguvėlė	small-scale	unknown	middle	4
09.08.2012	Smilgiai	small-scale	unknown	middle	3
					93

fungicides 4–7 times per season. Conventional farmers rotate fields and grow potatoes every 3–4 years. The experimental field plots were located at the Lithuanian Research Centre for Agriculture and Forestry in Akademija.

At most sampling sites, two to 14 isolates were cultured, with the exception of two sites at Krekenava in 2011 and at Latavėnai in 2012 where only one isolate was cultured (Table 1). A single lesion was collected from each plant. From the large scale grower fields and the experimental field plots, samples were collected at the beginning of late blight infection and at the middle of the outbreak (1–2 weeks later). From the small scale farms, samples were taken at the middle of the outbreak and at the end of the growing season (> 3 weeks later). Samples were not collected from small-scale farmers' fields at the beginning of late blight outbreak due to technical circumstances. In the early stages of the outbreak, approximately 10–15% of the leaf area of the infected plants and less than 10% of plants were infected with late blight. In the later stages, about 20–30% of the leaf area and more than 50% of the plants were infected. The plants were selected by randomizing the distance from field edges, and from each plant the blighted leaf was also randomly chosen, excluding those that had several or no lesions. Isolations were carried out and maintained using methods described by Runno-Paurson et al. (2009). All phenotypic tests were carried out in September–November of the year of isolation (Table 2).

Phenotypic analyses

Mating types of Lithuanian isolates (Table 1) were determined using the method described in Runno-Paurson et al. (2009). Observed oospore formation in single isolate pure cultures was interpreted as the occurrence of self-fertility in the isolates. The tester isolates were the same as those described in Lehtinen et al. (2007). Isolates forming oospores on plates with the A1 mating type were registered as A2; isolates that formed oospores with the A2 mating type were registered as A1.

The resistance to metalaxyl of all isolates was tested using a modification of the floating leaflet method (Hermansen et

Table 2: The number of isolates of *Phytophthora infestans* collected and tested for mating type, metalaxyl response, virulence phenotype during 2010–2012

| Year | Tested for | | |
	Mating type (n)	Metalaxyl (n)	Virulence (n)
2010	21	10	–
2011	42	34	40
2012	30	27	28
Total	93	71	70

al. 2000). Leaf disks (14 mm diameter) were cut with a cork borer from leaves of five-week-old greenhouse-grown plants. The susceptible cultivar 'Berber' was used. Six leaf disks were floated abaxial side up in Petri plates (50 mm diameter) each containing 7 ml distilled water or metalaxyl in concentrations of 10.0 or 100.0 mg l^{-1} prepared from technical grade metalaxyl-M (Syngenta experimental compound (metalaxyl-M), CGA 329351A). The inoculation and trial incubation was done as described by Runno-Paurson et al. (2009). The isolates were rated resistant if they sporulated on leaf disks in 100 mg l^{-1} metalaxyl (Hermansen et al. 2000). Those sporulating on leaf disks in a metalaxyl concentration of 10 mg l^{-1}, but not on leaves floating on 100 mg l^{-1} were rated intermediate, and those sporulating only in water were rated sensitive.

The pathotype of studied isolates was determined in 2011 and 2012 using Black's differential set of potato genotypes containing resistance genes R1-R11 from *Solanum demissum* (Malcolmson & Black 1966) (provided by the Scottish Agricultural Science Agency). Pathotypes were not tested in 2010 due to logistic reasons. Laboratory procedures were as described in Runno-Paurson et al. (2009). The virulence testing was done on detached leaflets.

Data analysis

Statistical analyses were performed with the SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC, USA). Differences in the prevalence of the two mating types of *P. infestans* isolates between years, study sites, regions and agricultural management practices were tested using a logistic analysis (GENMOD procedure in SAS) with a multinomial response variable (A1, A2, or both). Analogous logistic procedures were used to examine the differences in the resistance to metalaxyl (a multinomial response variable: resistant, intermediate or sensitive) between years, sites, regions, agricultural management practices, and also between different mating types. The dependence of specific virulence (percent of isolates that show virulence against particular R-genes) on years, sites, regions, agricultural management practices and R-genes was analyzed with type III ANOVA and Tukey HSD post-hoc tests (α = 0.05). In all analyses, "year" was treated as a categorical variable.

Race diversity was calculated with the normalized Shannon diversity index (Sheldon 1969). The dependence of race complexity on isolation time was analyzed with one-way ANOVA and Tukey HSD test, as were the differences in the Shannon index values between collecting years, sites and regions. To test for divergence of the A1 and A2 mating types from a 1:1 ratio, the χ^2 goodness-of-fit test was performed.

Results

The origin and characteristics of the *Phytophthora infestans* isolates collected in Lithuania during 2010–2012 are presented in Table 1; 93 isolates were analyzed for mating type, 71 isolates for metalaxyl response and 70 for virulence.

Mating type

Both A1 and A2 mating types were found in all three study years. Of the 93 isolates, 46 were A1 mating type, 42 were A2 mating type and five were self-fertile (Fig. 2). The overall mating type ratio did not deviate significantly from the expected 1:1 ratio (Chi-square = 0.09; *p* = 0.76) (Fig. 2). No significant differences were found in any of the study years (2010 – Chi-square = 0.09, *p* = 0.76; 2011 – Chi-square = 0.45, *p* = 0.051; 2012 – Chi-square = 0.30, *p* = 0.58). A1 mating type isolates were detected in 16 of 20 sampling sites, and A2 mating type isolates were identified in 14 sampling sites. Both mating types were present in nine of 20 sites (45%) where more than one isolate was tested. The proportion of A2 was 41% in 2010, 48% in 2011 and 50% in 2012, but there were no significant differences between collecting years (Chi-square = 3.56, df = 4, *p* = 0.47). There were considerable differences (Chi-square = 84.49, df = 44, *p* = 0.0002) in the proportion of A1 and A2 between sampling sites as well as between different regions (Chi-square = 14.79, df = 6, *p* = 0.022), with a higher proportion of A2 mating type in the northern (16 out of 21) and eastern regions (8 out of 19). There were no significant differences between different agricultural management practices (Chi-square = 3.11, df = 2, *p* = 0.54).

There was a statistically significant association between response to metalaxyl and mating type (Chi-square = 11.36, df = 4, *p* = 0.024). Of the 39 A1 mating type isolates, three were metalaxyl-resistant, three intermediate in resistance and 33 were sensitive. The 29 A2 mating type isolates tested included 10 resistant, five intermediate and 14 sensitive. Of the three self-fertile isolates, one was resistant and two were sensitive to metalaxyl (Fig. 3B).

Metalaxyl

Of the 71 isolates, 14 were resistant, 8 intermediate and 49 sensitive to metalaxyl (Fig. 3A). There were no significant differences between sampling years (Chi-square = 4.43, df = 4,
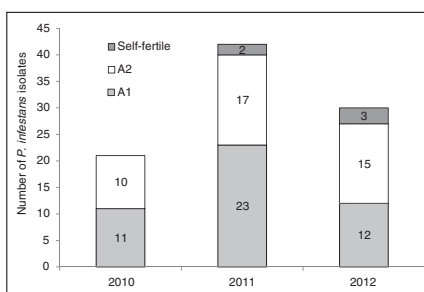

Fig. 2:   Mating type distribution of 93 isolates of *Phytophthora infestans* from Lithuania (2010–12).
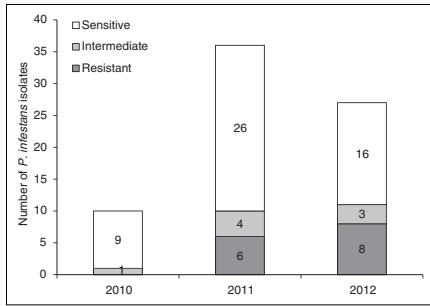
Fig. 3A: Metalaxyl sensitivity among isolates of *Phytophthora infestans* from Lithuania (2010–12).
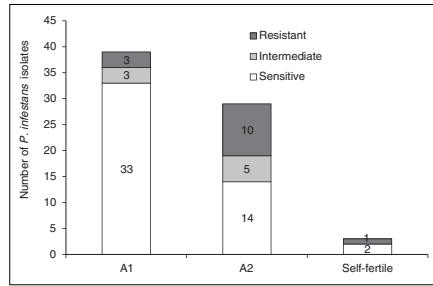

Fig. 3B: Metalaxyl sensitivity among isolates of *Phytophthora infestans* of A1, A2 and self-fertile mating type in Lithuania (2010–12).

$p = 0.35$). Significant differences were found between sampling sites (Chi-square = 43.64, df = 19, $p = 0.001$). The frequency of intermediate isolates was higher in northern and eastern parts and of sensitive isolates in central and south-western regions, but statistically significant differences between regions were not found (Chi-square = 11.36, df = 6, $p = 0.078$). There were no significant differences between different agricultural practices (Chi-square = 3.59, df = 4, $p = 0.46$), but there was a slight indication of more resistant isolates on field trials compared to large and small conventional growers' fields.

Pathotype

All 11 known virulence factors were found among the 70 tested isolates (Fig. 4). A significant difference in the prev-

alence of virulence factors was observed in the two sampling years ($F_{(10, 175)} = 28.36$, $p < 0.001$). Virulence factors 9 (2011: 8.6 ± 5.4%; 2012: 20.8 ± 16.3%), 5 (2011: 15 ± 6.7%; 2012: 24.33 ± 10.5%) and 8 (2011: 19.8 ± 8.8%; 2012: 15.3 ± 11.1%) were relatively rare (Fig. 4). No significant differences in virulence factors between years were found ($F_{(1,175)} = 2.44$, $p < 0.12$). There were no differences between agricultural practices ($F_{2,170} = 1.74$, $p = 0.18$) ors geographical regions ($F_{3,170} = 0.41$, $p = 0,75$) but there was a significant difference between different sites ($F_{16,160} = 3.88$: $p < 0,0001$).

There were high levels of diversity with 38 races among the 70 isolates tested (Table 3). The average number of virulence factors per isolate was 7.2 and ranged among sites from 5.2 to 10.3. Complex races predominated in both years being 7.2. Complex races were more frequently found among isolates collected from small-scale grower fields (7.5) than
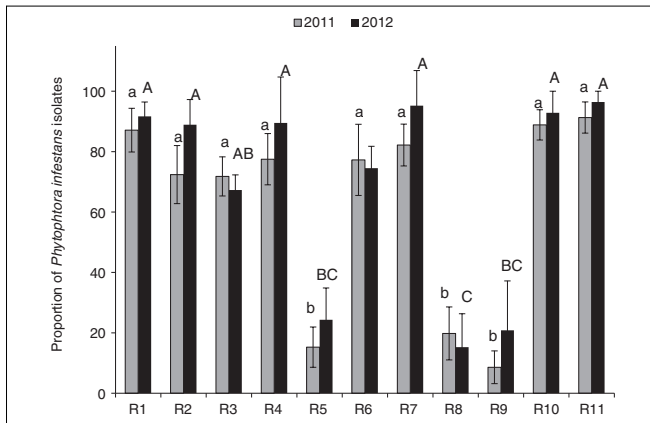

Fig. 4: Frequency of virulence to potato R-genes in the Lithuanian population of *Phytophthora infestans* in 2011 and 2012. Data are presented as means ± SE. Different letters upon the boxes indicate significant differences at $\alpha = 0.05$ (Tukey HSD test).

Table 3: Race frequencies among isolates of *Phytophthora infestans* from Lithuania (2011–2012)

Pathotype	Number of virulence factors	Number of isolates	
		2011	2012
1.10.11	3	1	
1.2.3.4.5.6.7.10.11	9	1	3
1.2.3.4.5.6.7.8.10.11	10	3	
1.2.3.4.5.6.7.8.9.10.11	11	4	3
1.2.3.4.5.6.7.9.10.11	10	2	
1.2.3.4.6.10.11	7	1	
1.2.3.4.6.7.10.11	8	3	6
1.2.3.4.6.7.8.10.11	9	2	
1.2.3.4.6.7.9.10.11	9		1
1.2.3.4.7.10.11	7	1	3
1.2.3.6.11	5	1	
1.2.3.6.7.10.11	7	3	
1.2.3.7.10.11	6		1
1.2.4.5.6.7.10.11	8		1
1.2.4.6.10.11	6	1	
1.2.4.6.7.10.11	7	1	2
1.2.4.6.7.8.10.11	8	2	
1.2.6.7	4	1	
1.2.6.7.10.11	6	1	
1.2.7	3		1
1.3.4.6.7.10.11	7	2	
1.3.4.6.7.8.9.10.11	9	1	
1.3.4.7.10.11	6	1	1
1.3.7.10	4	1	
1.4.6.7.11	5	1	
1.4.7.10.11	5		1
1.4.7.8.10.11	6	1	
1.6.7.10.11	5	1	
11	1		1
2.3.4.6.7.10.11	7	1	
2.3.4.6.7.11	6		1
2.3.6.7.10.11	6		2
2.4.11	3		1
2.4.6.7.11	5	1	
2.6.7.11	4	1	
3.4.6	3	1	
3.5.7.10	4	1	
3.6.7.10.11	5	1	
Number of isolates		40	30
Number of pathotypes		29	15

from trial fields (6.4). The most common races: 1.2.3.4.6.7.10.11, 1.2.3.4.5.6.7.8.9.10.11, 1.2.3.4.7.10.11 and 1.2.3.4.5.6.7.10.11, represented only 34% of the isolates, 36% of the pathotypes were found only once, and in 2011, even more – half of detected pathotypes were unique (Table 3). The overall normalized Shannon diversity index was very high, 0.79. The diversity index was higher in isolates from 2011 (0.87) than in 2012 (0.75). No significant differences in the values of the normalized Shannon diversity index between different years ($F_{(1,3)}$ = 4.22, p = 0.18) or between different regions ($F_{(1,3)}$ = 0.54, p = 0.69) were found.

Discussion

This paper is the first report of finding the A2 mating type in the Lithuanian population of *P. infestans*. Our study indicates that the population is characterized by high levels of pathotypic diversity, the occurrence of A1 and A2 mating types and the prevalence of metalaxyl sensitivity.

Both mating types were found in Lithuania in all study years. The average percentage of the A2 mating type in 2010–12 was 45%. The frequency of A2 mating type remained quite stable during the three years, varying from 41% (17 out of 42) in 2011 to 50% (15 out of 30) in 2012. Considerable differences were found in the frequency of A1 and A2 mating types between sites and regions in Lithuania. The A2 mating type was more frequent in the northern region of Lithuania than in the eastern, central and south-western regions. These findings of mating types in *P. infestans* Lithuanian populations are comparable to those found in studies from Poland in 2006 and 2009 (Chmielarz et al. 2014), in Finland 2006−10 (Hannukkala 2012), other Nordic populations (Denmark, Norway, Sweden) in 2003 (Lehtinen et al. 2008), in the Russian Federation (Moscow region) 2007−09 (Statsyuk et al. 2013), in Estonia 2004−07 (Runno-Paurson et al. 2014) and the Czech Republic in 2003−05 (Mazakova et al. 2006). There are several *P. infestans* populations in Europe, in the UK, France (2006−07), Switzerland (2006−07), Belgium (2007), Denmark (2006−07), where A2 mating type frequency has increased to extremely high levels (Gisi et al. 2011) compared to previous studies. Undoubtedly, these findings are related to the dramatic changes that have occurred in *P. infestans* European populations, including in the UK. The extent of the A2 mating type has increased from very low (3−5%) up to 75% (Cooke et al. 2012) among the UK population since about 2004 very rapidly due to invasive lineage 13_A2 (also known as "Blue 13"). The possible evidence for 13_A2 in Lithuanian populations of *P. infestans* is presumed and should be shown in the near future.

Available evidence from northern Europe strongly supports regular sexual reproduction of *P. infestans* in Nordic and the north-east regions (Runno-Paurson et al. 2009, 2010a, 2014; Kuznetsova et al. 2010, Yuen and Andersson 2013). As the mating type ratio found among *P. infestans* populations in Lithuania in 2011 and 2012 was almost 1:1 and in 2010 did not diverge significantly from 1:1, and also both mating types were found in three years from half of the studied sites, we conclude that sexual reproduction probably takes place in the Lithuanian population of *P. infestans*. The presence of both mating types in the same potato field indicates the possibility of sexual reproduction of *P. infestans*, and possible contamination of soil with long-lived oospores (Turkensteen et al. 2000). On the question of the presence of oospores in certain regions, crop rotation is an important control measure against late blight and must be applied (Yuen & Andersson 2013). The normal crop rotation between

potatoes growing in Lithuania is on average three years. In small-scale farm fields (including small household gardens) rotation could vary from three years to shorter periods or even none. To account for oospores, rotation should be at least four years. In general, Lithuanian potato growers understand the need for crop rotation, so the possibility of oospores as initial late blight inoculum is still presumably smaller than in the case of northern populations due to climate and narrow crop rotation in potato production (Hannukkala 2012). However, elsewhere in Europe the role of oospores is not very significant and the major sources of primary inoculum are seed, volunteer plants and waste piles (Cooke et al. 2011).

Strong relationships between A2 mating type and metalaxyl-resistant isolates, and between A1 mating type and sensitive isolates were found in the present study. In the 1990s, metalaxyl resistance was mostly associated with A1 mating type in most of European *P. infestans* populations (Hermansen et al. 2000, Day et al. 2004, Cooke et al. 2006, Lehtinen et al. 2007). More recent studies have found a relationship between A2 mating type and metalaxyl-resistance in several populations (Cooke et al. 2011, Mazakova et al. 2011). However Gisi et al. (2011) studied *P. infestans* isolates collected from France, UK, Switzerland, Denmark, Netherlands, Belgium, Germany and Sweden and noticed that there was no genetic link between mating type and phenylamide (metalaxyl-M) resistance. Obviously A2 mating type and phenylamide resistance are associated in recent isolates by coincidence (Gisi et al. 2011).

Metalaxyl was introduced to the Lithuanian market in 1981 (Valskytė 1994). After using metalaxyl fungicide for a while, it became less effective, and a resistance problem was found (Valskytė 1994). In the early and middle 1990s, more than 70% of the isolates were resistant to metalaxyl in Lithuania (Valskytė 2000). Over a seven-year research period, the frequency of metalaxyl-resistant isolates fell from 98% in 1990 to 23% in 1998 (Valskytė 2000). The very high level of metalaxyl-resistance in 1990–95 was caused by intensive use of metalaxyl-containing preparations, because, at that time, only systemic fungicides of the phenylamide group were marketed (Valskytė 2000).

In the present study 2010–12, the situation is quite similar to that found in Lithuania at the end of the 1990s, where the sensitive isolates prevailed (70%) in all studied strains. Except in 2010, when none of the metalaxyl-resistant isolates were found and there were no differences found between different years and regions. The Lithuanian population of *P. infestans* contains a high sensitivity to metalaxyl. The product Ridomil Gold MZ 68WG contains metalaxyl-M and mancozeb, and is moderately used by large-scale potato growers in Lithuania (A. Ronis personal observation). Still, the majority (93%) of Lithuanian potatoes are grown by small-scale growers in fields of less than 5 hectares (Ronis et al. 2007). The financial resources of these farms are very limited, so use of chemical control for potato late blight or protective fungicides are a last resort.

Our research findings about the dominance of metalaxyl-sensitive strains in the Lithuanian population of *P. infestans* concur with those from other Northern-European countries,

such as Finland, Sweden, Denmark and Norway (Lehtinen et al. 2008), and Estonia (Runno-Paurson et al. 2014), as well as the Moscow region of Russia (Statsyuk et al. 2013), Belarus (Pobedinskaya et al. 2011) and Poland (Chmielarz et al. 2014). However, they differ completely from findings from France in 2006–07, and the UK in 2006–07 (Gisi et al. 2011), where the frequencies of metalaxyl-resistant isolates are very high. The frequency of metalaxyl-resistant isolates of *P. infestans* in the 2000 s in Nordic countries has been quite rare in spite of moderate usage of metalaxyl fungicides (Lehtinen et al. 2008). However, there are populations where metalaxyl-resistant strains remain at a low level, but with occasional seasonal fluctuations (Mazakova et al. 2011, Runno-Paurson et al. 2011, Chmielarz et al. 2014) considered as normal behaviour within *P. infestans* populations (Cooke et al. 2011).

The Lithuanian race structure studied in 2011–12 was highly diverse and complex. Most found races were unique, appearing only once, and the four most common pathotypes comprised only 34% of the population. The average number of infected Black's differentials per isolate in Lithuania was very high (7.2), similar to populations from eastern Europe (Śliwka et al. 2006, Statsyuk et al. 2013, Runno-Paurson et al.)

The high race variation calculated by the normalized Shannon diversity index within the Lithuanian population probably indicates high diversity of potato genotypes from which isolates were obtained, also apparently influenced by the distinctness of the agricultural management practises implemented on a large area of the potato production in Lithuania. The Lithuanian potato growing management contrasts notably from others in Europe. More than 90% of the potato production area is grown by small growers; on these potato fields old and late blight infected seed material is planted and the crops are not protected with late blight fungicides (Ronis et al. 2007). The phytosanitary quality of seed potatoes plays an important role in the amount of initial late blight inoculum. There is a direct connection between uncertain seed material and high phenotypic diversity of *P. infestans* (Bouws & Finckh 2007, Runno-Paurson et al. 2013b). By contrast in the large scale conventional productions, which account for less than 10% of all potato fields in Lithuania, farmers use high-quality certified seed potatoes and apply fungicides 4–7 times per season (Ronis et al. 2007).

The two most common races among Lithuanian isolates were 1.2.3.4.6.7.10.11 and 1.2.3.4.5.6.7.8.9.10.11; this is not common for other European populations (Hermansen et al. 2000, Knapova & Gisi 2002, Lehtinen et al. 2007, Lehtinen et al. 2008, Runno-Paurson et al. 2009, 2014). The prevailing race of *P. infestans* in most European populations is 1.3.4.7.10.11 (Hermansen et al. 2000, Knapova & Gisi 2002, Lehtinen et al. 2007, Lehtinen et al. 2008, Hannukkala 2012, Runno-Paurson et al. 2014), which was found as only one isolate over both years in Lithuania. Complex races are common in Polish populations (Śliwka et al. 2006, Chmielarz et al. 2014), a similar situation to those in Russia (Statsyuk et al. 2013) and in Estonia (Runno-Paurson et al. 2009, 2010a, 2014). The mean number of virulence factors per isolate

increased from 6.3 in 2002−03 to 6.9 in 2004−07. A similar increase has also occurred in Finland and Norway (Lehtinen et al. 2008, Hannukkala 2012). However, these findings may have been influenced by differences in virulence testing between different European laboratories, especially to R2, R5 and R9 which have been detected more frequently from northern Europe (Andrivon et al. 2011).

From our study, it appears that the type of agricultural management practice has no influence on the population structure of *P. infestans* in Lithuania. Obviously our research results were influenced by our late blight sampling strategy. While the majority of cultured isolates originated from small-scale farm fields, it reflects the direct situation in these populations. In other studies a direct connection between management practices has been found (Bouws & Finckh 2007, Lehtinen et al. 2008, Runno-Paurson et al. 2010).

The potato growing area in Lithuania has been decreasing year by year, mostly because many traditionally-managed family farms have disappeared (Ronis et al. 2007). This decrease is expected to continue into the near future with a corresponding increase in the number of mass producers, who will be able to change management practices including a shift from use of uncertified seed material to certified and protection of potato haulms against late blight until the end of the growing season (Ronis et al. 2007). All these factors should reduce the possibility of early occurrence of late blight and should allow efficient control of late blight. Increased sexual reproduction leads to increased variability in the pathogen population, with immediate implications for the use of host-plant resistance and fungicides (Yuen & Andersson 2013).

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CURRICULUM VITAE

First name: Alice
Surname: Aav
Date of Birth: 07.05.1985
E-mail: alice.aav@emu.ee

Academic degree:

2011	Master's degree (MSc) "Late blight resistance of potato varieties and impact of sexual reproduction of *Phytophthora infestans* to potato production"

Education:

2011–2016	Estonian University of Life Sciences, *PhD* studies in Plant Pathology
2009–2011	Estonian University of Life Sciences, *MSc* studies in horticulture/plant protection (*Cum laude*)
2004–2009	Estonian University of Life Sciences, *BSc studies* in horticulture
1999–2004	Suure-Jaani Gymnasium
1994–1999	Võhma Gymnasium
1993–1994	Villevere Primary School
1992–1993	Paide Gymnasium

Foreign languages: English, Finnish, Russian

Professional Employment:

2011–2014	Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, specialist
2010–2011	Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, technician

Target financed projects and grants of Estonian Science Foundation:

2008–2011	Project ETF7391 "Foraging behaviour of pollinators in farmland: impact of agricultural practices". Senior personnel
2009–2014	Project SF0170057s09 "Plant protection for sustainable crop production". Senior personnel
2012–2015	Project ETF9432 "Phenotypic and genotypic characterisation of Baltic and Russian Pskov region populations of

Phytophthora infestans; the role of oospores as a source of primary inoculum to late blight pathogen epidemiology". Senior personnel

Other projects:

2011–2015   Project AR11121 "Breeding for disease resistance in plants". Other staff

2011–2015   Project 8-2/T12004PKTK "RESIST project breeding for disease resistance in plants" Research staff

Research interests:

Phenotypic characterisation of the late blight pathogen *Phytophthora infestans* populations of Baltic States, Plant Pathology.

Honours and Awards:

2011   DoRa T8 – "Participation of young researchers in the international circulation of knowledge", scholarship from Archimedes Fund

2010   Rotalia Foundation (USA) scholarship

2010   Scholarship of the Estonian World Council, Inc.

Professional training:

2015   EPOS: Organic Food Production Chain (Summer Course in Warsaw University of Life Sciences, Poland)

2011   qPCR Experience Real-Time PCR in Plant Pathology: Diagnostics and Research (National Institute of Biology, Slovenia)

2011   Hands in using PCR (certificate no. 8-19/47, Saku, Estonia)

Administrative responsibilities:

2014– …   Member of Christian Congregation Risttee in Tartu

2012– …   Member of Estonian Green Movement FoE

2012– …   Member of Tartu Organic Garden FoE

2010– …   Member of Estonian Naturalists' Society

2010– …   Member of Horticultural Society Kanarbik

2010– …   Member of Estonian Plant Protection Society

2002–2014   Member of Estonian Evangelical Lutheran Church in Suure-Jaani Congregation

ELULOOKIRJELDUS

Nimi: Alice Aav
Sünniaeg: 07.05.1985
E-mail: alice.aav@emu.ee

Akadeemiline kraad:
2011   Magistrikraad (*MSc*) „Kartulisortide lehemädanikukindlus ja *Phytophthora infestans* sugulise paljunemise esinemise mõju kartulikasvatusele"

Hariduskäik:
2011–2016   Eesti Maaülikool, doktorantuur fütopatoloogia erialal
2009–2011   Eesti Maaülikool, magistrantuur aiandus/taimekaitse (*Cum laude*)
2004–2009   Eesti Maaülikool, bakalaureus aianduse erialal
1999–2004   Suure-Jaani Gümnaasium
1994–1999   Võhma Gümnaasium
1993–1994   Villevere Algkool
1992–1993   Paide Gümnaasium

Emakeel: Eesti

Võõrkeeled: Inglise, soome, vene

Erialane teenistuskäik:
2011–2014   Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, spetsialist
2010–2011   Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, tehnik

Osalemine uurimisprojektides:
Sihtfinantseerimine ja grandid:
2008–2011   Grant ETF7391 „Tolmeldajate korjekäitumine põllumajandusmaastikes: põllumajandusliku tegevuse mõju". Põhitäitja
2009–2014   Sihtfinantseeritav project SF0170057s09 „Taimekaitse jätkusuutlikule taimekasvatusele".
2012–2015   Grant ETF9432 „*Phytophthora infestans* Baltikumi ja

Venemaa Pihkva regiooni populatsioonide fenotüübiline ja genotüübiline iseloomustamine; oospooride kui esmase nakkusallika roll lehemädanikutekitaja epidemioloogias". Põhitäitja

Muud projektid:
2011–2015   SA Archimedes, Euroopa Regionaalarengu Fond R11121 „Põllukultuuride resistentsusaretus". Täitja
2011–2015   Struktuuritoetus, Meede „Biotehnoloogia teadus- ja arendustegevuse toetamine" projekt 8-2/T12004PKTK „Põllukultuuride resistentsusaretus". Põhitäitja

Teadusliku uurimustöö suunad:
Kartuli-lehemädaniku tekitaja *Phytophthora infestans* Baltikumi populatsioonide fenotüübiline iseloomustamine, fütopatoloogia.

Teaduspreemiad ja -tunnustused:
2011   SA Archimedes, DoRa T8 – „Noorteadlaste osalemine rahvusvahelises teadmisteringluses" stipendium
2010   Rotalia Fondi (USA) stipendium
2010   Ülmaailmse Eesti Kesknõukogu stipendium.

Täiendkoolitused:
2015   EPOS: Mahetoidu tootmisahel (Suveülikooli kursus Varssavi Põllumajandusülikoolis, Poolas)
2011   qPCR kasutamine fütopatoloogias (Riiklik Bioloogia Instituut, Sloveenia)
2011   PCR kasutamise koolitus (sertifikaat nr. 8-19/47, Saku, Eesti)

Organisatsiooniline tegevus:
2014– …   Tartu Kristliku Risttee Koguduse liige
2012– …   MTÜ Eesti Roheline Liikumine liige
2012– …   MTÜ Tartu Maheaed liige
2010– …   Eesti Looduseuurijate Seltsi liige
2010– …   Võhma aiandusselts Kanarbik liige
2010– …   Eesti Taimekaitse Seltsi liige
2002–2014   Eesti Evangeelse Luterliku Kiriku Suure-Jaani Koguduse liige

LIST OF PUBLICATIONS

1.1. Publications indexed in the ISI Web of Science database:

Runno-Paurson, E., Kiiker, R., **Aav, A.**, Hansen, M., Williams, I. H. 2016. Distriution of mating types, metalaxyl sensitivity and virulence races of *Phytophthora infestans* in Estonia. Agronomy Research, 14 (1), 220–227.

Aav, A., Skrabule, I., Bimšteine, G., Kaart, T., Williams, I. H., Runno-Paurson, E. 2015. The structure of mating type, metalaxyl resistance and virulence of *Phytophthora infestans* isolates collected form Latvia. Zemdirbyste-Agriculture, 102 (3), 335–342.

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Runno-Paurson, E., Remmel, T., Ojarand, A., **Aav, A.**, Mänd, M. 2010. The structure of *Phytophthora infestans* populations from organic and conventional crops. European Journal of Plant Pathology, 128 (3), 373–383.

3.5. Articles/presentations published in Estonian conference proceedings:

Runno-Paurson, E., **Aav, A.**, Einola, A., Vaater, V. 2011. Kartuli-lehemädaniku vaatluskatse tulemustest 2010 Einola talus. Agronoomia 2010/2011, 197–202. AS Rebellis.

5.2. Conference abstracts that are not indexed in ISI Web of Science:

Aav, A., Runno-Paurson, E., Nassar, H., Hansen, M., Kotkas, K., Liiv, K., Skrabule, I., Bimšteine, G., Williams, I. H., Mänd, M. 2013. Phenotypic characterisation of Latvian isolates of *Phytophthora infestans* in 2010–2011. *Book of abstracts Down to Earth,* Tallinn University, Estonia, May, 2013.

VIIS VIIMAST KAITSMIST

HIIE IVANOVA

RESPONSES OF RESPIRATORY AND PHOTORESPIRATORY DECARBOXYLATIONS TO INTERNAL AND EXTERNAL FACTORS IN C_3 PLANTS

RESPIRATOORSE JA FOTORESPIRATOORSE DEKARBOKSÜÜLIMISE VASTUSED SISEMISTE JA VÄLISTE FAKTORITE TOIMEL C_3 TAIMEDELE

Professor **Ülo Niinemets**, vanemteadur **Tiit Pärnik**, vanemteadur **Olav Keerberg**

08. jaanuar 2016

DIEGO SANCHEZ DE CIMA

SOIL PROPERTIES AFFECTED BY COVER CROPS AND FERTILIZATION IN A CROP ROTATION EXPERIMENT

VAHEKULTUURIDE JA VÄETAMISE MÕJU MULLA OMADUSTELE KÜLVIKORRAKATSES

Dotsent **Endla Reintam**, emeriitprofessor **Anne Luik**

11. veebruar 2016

KRISTI PRAAKLE

CAMPYLOBACTER SPP. AND *LISTERIA MONOCYTOGENES* IN POULTRY PRODUCTS IN ESTONIA

CAMPYLOBACTER SPP. JA *LISTERIA MONOCYTOGENES* LINNULIHATOODETES EESTIS

Professor **Mati Roasto**, professor **Marja-Liisa Hänninen** (Helsingi Ülikool), professor **Hannu Korkeala** (Helsingi Ülikool)

04. märts 2016

TARMO KALL

VERTICAL CRUSTAL MOVEMENTS BASED ON PRECISE LEVELLINGS IN ESTONIA

MAAKOORE VERTIKAALLIIKUMISED EESTIS TÄPPISNIVELLEERIMISTE ANDMETEL

Emeriitprofessor **Jüri Randjärv**, dotsent **Aive Liibusk**

29. aprill 2016

ELSA PUTKU

PREDICTION MODELS OF SOIL ORGANIC CARBON AND BULK DENSITY OF ARABLE MINERAL SOILS

MINERAALSETE PÕLLUMULDADE ORGAANILISE SÜSINIKU JA LASUVUSTIHEDUSE STATISTILISED PROGNOOSIMUDELID

Professor **Alar Astover**, dotsent **Christian Ritz** (Kopenhaageni ülikool)

14. juuni 2016