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The fight against *Phytophthora infestans* to keep potato plants healthy

Bachelor Thesis Biology

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Photo front page: Symptoms of late blight disease. *Source: aps.net*

Summary

The potato (*Solanum tuberosum*) is one of the most consumed crops in the world and it is of major importance for both developed and developing countries. Unfortunately, the crop is affected by a devastating disease called late blight that can quickly kill entire potato fields. This results in enormous economic and environmental problems. Regrettably, problems with late blight seem to increase in recent decades. In this bachelor thesis detailed information will be presented about late blight and how to control it. How is knowledge about the interactions between pathogen and potato plant used to control late blight disease? Are there any recommendations for a better control of late blight?

The cultivated potato species originated in South America, were many other wild potato species live. Potato cultivars have a very low genetic diversity due to strong inbreeding, which makes the crop susceptible for epidemic diseases, including late blight.

Late blight is caused by the oomycete species *Phytophthora infestans.* After its introduction in Europe in the 1840s, it caused several widespread famines, the most infamous being the Great Irish Famine, which resulted in the death of one million people. The pathogen has an enormous capable of adapting itself to changing environments and it can easily become resistant against late blight control strategies. *P. infestans* can reproduce both asexually and sexually. Asexual reproduction via zoosporogenesis contributes greatly to the quick spread of late blight in the growing season, since it generates a lot of motile shorted-lived zoospores that can all infect new plant tissues. Historically, *P. infestans* reproduced asexually in most parts of the world, because only one mating type was present. A few decades ago another mating type spread across the world, making sexual reproduction possible. Sexual reproduction via oosporogenesis results in the production of thick-walled oospores that can survive long times in the soil, contributing to the survival of *P. infestans* during the winter and it generates new genetic diversity due to recombination. Sexual reproduction is the major cause of the increasing problems with controlling late blight in recent decades.

Potato plants defend themselves against *P. infestans* by using their immune system. Pathogens try to manipulate that immune system by secreting effector molecules in the plant. Plants can in turn use resistance genes to recognize effector molecules, making the plant resistant against the pathogen. As a result, there is an ongoing arms race between potato plants and pathogens. Cultivated potatoes possess not many resistance genes, making them highly susceptible to late blight.

To control late blight, an integrative approach is used with the goal to prevent late blight from occurring and spreading: multiple control strategies are always combined. Fungicides are sprayed on the potatoes during periods when chances of infection are high, though there is a need to diminish the use of fungicides. The use of living organisms, ground coverage and proper winter storage of potatoes are examples of other control strategies. Breeding resistant plants is always a part of an integrative approach: there are different ways to breed durably resistant plants.

Current late blight control programs are generally well organized. Farmers know what to do to prevent late blight from occurring. To further improve the control of late blight, it is recommended to gain more knowledge about the life cycle and the infection process of *P. infestans* and to study late blight in a more interdisciplinary way. Besides, efforts should be made to internationalize the control of *P. infestans*. This will help to better control late blight in the future.

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1. Introduction

Feeding a future world with nine billion people in 2050 seems to be a difficult task, especially if one considers that nowadays already more than 800 million people are undernourished. Food production certainly has to increase, while food losses have to decrease. Importantly, this all should be done in a sustainable manner that takes in account amongst others climatic change and biodiversity issues. A new green revolution in agriculture is thus quickly needed (Beddington, 2010). The potato (*Solanum tuberosum*) has a huge potential to attribute to this revolution: it has a high nutritional value and can be grown almost everywhere. It is the third most consumed crop in the world by human, after wheat and rice (Haverkort *et al.*, 2009). Developing countries showed a three-fold increase in the area cropped with potatoes from 1960 to 2008, while in the same period the area in developed countries decreased by half (Haverkort *et al.*, 2009). In 2005, a total area of almost 20 million hectares was cropped with potatoes producing 300 Mt of potatoes (the total area of land on earth is 150 million hectares). European people still ate most potatoes of all people in the world in 2005: almost 89 kilograms per capita were consumed in that year. In the same year, Dutch people ate 86 kilograms of potatoes per capita of which 53 kilograms was consumed as an unprocessed product. The Low Countries (the coastal region in north western Europe) are major producers of potatoes in Europe and even in the world: the Netherlands were in 2007 the 9th most producing country in the world (Haverkort *et al.*, 2009). This is a rather notable fact since all other countries in the top ten are several times larger than the Netherlands. In 2007 (raw) potato production in the Netherlands was worth 787 million euros, thereby being of major economic importance (Haverkort *et al.*, 2008). When these potatoes are processed to products like chips, they represent a value of 3 billion euros. More than 11.000 farmers grow potatoes and there are many more people working in the whole potato sector.

Nevertheless, using the potato to feed the world in a sustainable way is made difficult by a deadly pathogen: the oomycete *Phytophthora infestans* causes late blight disease in potatoes, which is the most important and devastating disease in that crop (Haverkort *et al.*, 2008). Two to four days after a potato plant is infected with *P. infestans* the first symptoms of late blight become visible: small purple or dark-green lesions often surrounded by a yellow ring that quickly become brown appear on the leaves (Fig. 1A,B; Christ, 1998; Fry, 2008; Nowicki *et al.,* 2012). Stems can also be infected and show the same symptoms as leaves, though lesions are darker. Moreover, tubers can be infected. Tuber infection is characterized by the presence of slightly depressed brownish or purplish lesions on the surface (Fig. 1G). When the tuber is cut at the surface of the lesions, reddish-brown rotten areas that extend to the interior of the tuber are visible. In the beginning, the lesions on leaves are only one or two millimeters in diameter. The lesions quickly enlarge and a white fuzzy fungus-like growth can often be observed at edge of the lesions after four to six days when weather conditions are cool and humid (Fig. 1C). Due to the progressive enlargement of the lesions, leaves can turn completely brown and dried out in a few days: they will die quickly (Fig. 1D). The disease can spread quickly from one part of the plant to another part: it can spread from the sprout to the tuber and vice versa. Moreover, the disease can spread to other potato plants. Within seven to ten days, a whole plant can be killed and within two to three weeks a complete field of potato crops can be killed (Fig. 1E,F).

The diseases can be controlled to a certain degree by applying fungicides to the crops. The production of these fungicides is nevertheless costly in both environmental and economic ways (Haverkort *et al.*, 2008). Economic losses due to late blight in the Netherlands are approximately 124 million euros per year (15.8% of national raw potato production). In the European Union, approximately one billion euro per year is lost due to the disease, while this is approximately 10 billion euros per year for the world as a whole (7.5% of global raw potato production). Developing countries account for most of these losses, since farmers there often do not have access to proper fungicides. In the Netherlands, approximately 14.5 kg of carbon dioxide is produced when synthesizing one kg of fungicide and 12% of the energy the potato sector uses, is used to control the

disease, thereby contributing to the major environmental problems the world faces (Haverkort *et al.*, 2008). Moreover, epidemic outbreaks of the disease in developing countries can result in local famines and other human disasters that can in turn result in the death of many people. Economic and environmental problems associated with late blight are expected to increase in the nearby future (Haverkort *et al.*, 2009). Problems with late blight were always large, but they have become much larger in recent decades (Fry, 2008). Overall seen, management techniques to prevent late blight from occur are working less and less well, resulting in the loss of more potatoes and sometimes an intensification of fungicide spraying. The pathogen is becoming resistant against fungicides and previously resistant cultivars are no longer resistant anymore (Nowicki *et al.,* 2012). It is thus of major importance to find effective and sustainable ways to combat late blight.

This bachelor thesis will give detailed information about late blight and focuses on methods to deal with late blight. How is knowledge about the interactions between *Phytophthora infestans* and potato plant used to control late blight disease? Are there any recommendations to make for a better control of late blight? After giving some background information on the potato plant, the origin, spread and life cycle of the oomycete pathogen *Phytophthora infestans*, the organism that causes late blight, will be discussed in detail. It will become clear why late blight problems are becoming more severe nowadays. Plants can defend themselves against pathogens, so subsequently detailed information will be provided on how potato plants and *P. infestans* interact during an infection with late blight. All this knowledge is used to design management strategies to address late blight. These strategies will be discussed, with a focus on resistance breeding. Finally, an evaluation of management strategies and current research focuses will be provided.

Fig. 1. Symptoms of late blight disease. The disease can quickly kill entire plants and, moreover, it can kill entire fields with potatoes if no control strategies are used. *Source: aps.net*

2. Potato plants: plants that conquered the world

The potato plant (*Solanum tuberosum*) is a perennial plant that is part of the nightshade family (*Solanaceae*). The shoots op the plants can grow as high as sixty centimeters and it can have different colors of flowers. The plant can reproduce sexually through pollination mediated by bumblebees, but it can also reproduce asexually. After flowering, small green fruits are formed, which are toxic due to high concentrations of solanine. The plants reproduce asexually through the formation of potato

tubers, which are the consumable part of the plants. These tubers develop from so-called stolons (Fig. 2). These are parts of the stem that grow underground and look like horizontally growing thickened roots. The tip of a stolon can develop in a tuber under the influence of various exogenous and endogenous signals (Fernie & Willmitzer, 2001). High gibberellic acid, high cytokinin, high jasmonates and high sucrose concentrations stimulate tuber formation, while the process is also stimulated by short periods of daylight (probably mediated by low concentrations of phytochrome, a photoreceptor), low nutrient availability and low temperatures. During tuber formation, the metabolism and physiology of the stolon cells that develop into a tuber changes dramatically (Fernie & Willmitzer, 2001). The stolon cells grow larger and become storing places for proteins, but above all for carbohydrates. Mainly sucrose is transported to the cells that develop into the tuber and this is mainly stored as starch in amyloplasts. The transcription of many genes changes during the tuber-formation process and transcriptomic analyses revealed that the transcription of proteinase inhibitors and patatin, the main storage protein in the tuber, was upregulated by more than 5-fold (Xu *et al.*, 2011). Besides, genes that function in starch synthesis were upregulated more than 4-fold. While the shoot dies during a cold period, the tubers stay alive, remaining dormant. When the cold period is over, the tuber starts sprouting to form a new plant. Sprouting seems to be stimulated by higher temperatures, higher gibberellic acid, lower abscisic acid and possibly also by very high cytokinin concentrations (Fernie & Willmitzer, 2001). During sprouting the transcription of genes changes again, with genes that are involved in starch breakdown being expressed more.

The asexual life cycle is of major importance in agriculture, since tubers are the parts of the plant that are consumed. Moreover, asexual reproduction is used in agriculture to not loose valuable traits: it results in the formation of genetically identical clones of the mother plant. In agriculture, three types of potato tubers can be distinguished (Haverkort *et al.*, 2008). Potato tubers used to make the next generation of plants are called seed potatoes. These are stored during winter to prevent too early sprouting and are sown the next spring. Tubers that are used for direct consuming are called ware potatoes. Starch potatoes are processed in different starch containing products. Since these three types of potatoes need to have different traits for performing well, lots of potato cultivars (more than 4000) have been bred, amongst which Bintje and Eigenheimer are very famous. These cultivars are derived from only a very small number of plants that were mainly propagated asexually. A complete cultivar is often a genetic clone from one single parent plant with desired traits. Breeders often try to breed potato plants that are homozygous for a desired trait for practical reasons. When homozygous plants reproduce sexually, the trait is not lost in a fraction of the offspring. Cultivated potatoes have as a consequence of these things a very low genetic diversity: they are strongly inbred (Xu *et al.*, 2011). This makes cultivated potatoes very susceptible for pests

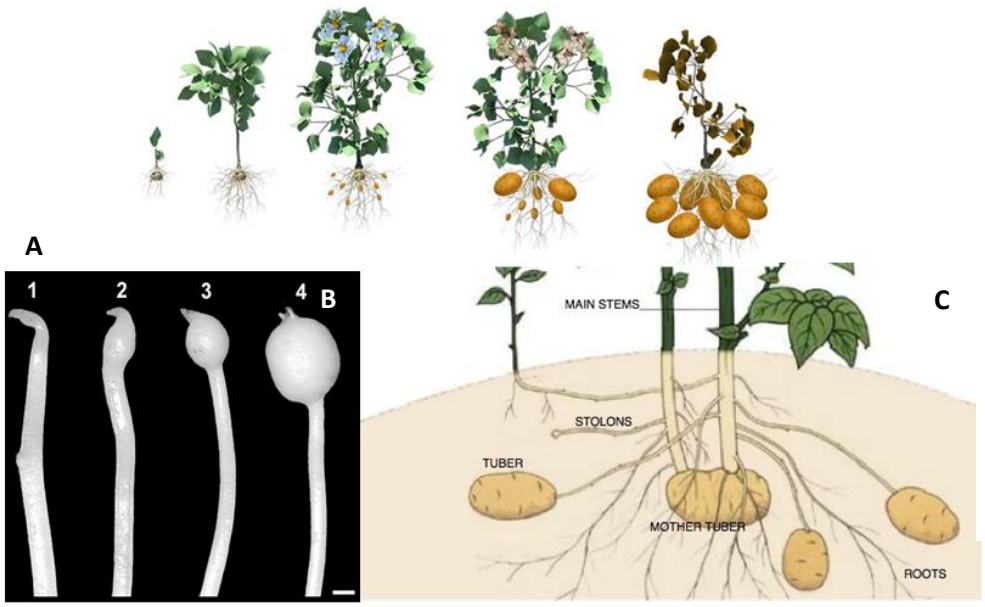

Fig. 2. A: The asexual life cycle of the potato plant. Potato tubers are the overwintering structures of the plant. *Source: www.ruralliquidfertilisers.com* **B**: The progressive development of a tuber from the tip of a stolon (Fernie & Willmitzer, 2001). **C**: Stolons are thickened underground parts of the stem from which new, genetically identical plants or tubers can grow. They are thus no roots. *Source: potatoes.co.nz*

and diseases. As a result of constant asexual reproduction, potato plants have no mechanism to get rid of deleterious alleles, so these accumulate (Xu *et al.*, 2011). This phenomenon is known as inbreeding depression. The genome of the potato is filled with mutations that disrupt normal transcription of genes. This makes the potato plant even more susceptible for pests and diseases and decreases the yield of a plant.

2.1 The origin of the potato and its spread over the world

Genetic studies revealed that the species *Solanum tuberosum* originated in the South American Andes due two polyploidization events that occurred more than 60 million years ago (Xu *et al.*, 2011). The cultivated potato is tetraploid, has 48 chromosomes and is heterozygous for a lot of traits. Besides the worldwide cultivated species *Solanum tuberosum*, 187 other wild tuber bearing *Solanum* species have yet been discovered in the Andes, ranging from Chile to Texas in the USA (Hijmans *et al.*, 2007). The ploidy of these species ranges from diploids to hexaploid, though most species are diploid. It is thought that polyploidization is a very important mechanism by which new potato species have arisen in the Andes and that it has contributed to the ecological differentiation between species (Hijmans *et al.*, 2007). The sprouts of most wild species look very much like the cultivated potato, though the tubers are often very different and not eatable (Fig. 3). Potato species are often found in tropical highlands at heights between 2000 and 4000 meter and most species are very rare and have a very narrow range in which they appear (Hijmans & Spooner, 2001). Potato species richness is not evenly distributed through the Andes: the distribution is rather patchy. Central Mexico has very high species richness, but Southern Peru has the highest species richness and does also harbor the highest number of rare species (Hijmans & Spooner, 2001). Around 3000 BC, it was in Southern Peru, in the Altiplano, that the Incas started the cultivation and domestication of the potato, but even before 3000 BC, people in Chile probably gathered and ate wild potatoes (McNeill, 1999). The Incas did not only cultivated *Solanum tuberosum*, but also several other potato species. Nowadays, still six other potato species are cultivated solely in South America. After Columbus discovered America, the Spanish quickly established colonies in South America and came into contact with the Incas and the potato. The Spanish have probably taken the potato species *Solanum tuberosum* on board of their ships when they left South America to sail home again, because they needed food on that journey. Back in Spain, the Spanish quickly distributed the potato throughout Europe, because they had a huge empire at that time. Around 1600 the potato was present in the Low Countries and botanists described it: the famous Carolus Clusius found it a very special plant. The potato was planted in botanical gardens and used as a medicine for several diseases and as status symbol for the upper class people. Driven by intense hunger, some very poor people quickly started cultivating the potato in small gardens, since its tubers had a high nutritional value, but grain long remained by far the most cultivated crop for more than a century. The potato was thus not eaten at a large scale. In that time, people were often afraid to eat potatoes, because it was thought that all kinds of evil might happen to you when you eat potatoes and stop eating normal things like grain. Nevertheless, from 1750 onwards the potato was started to be cultivated at a large scale in Europe, partly due to famines caused by epidemic diseases in grain crops. Governments started to stimulate people to grow potatoes and eat them, because that would save countries from famines, which could save the life of many people. This

Fig. 3. Wild potato species differ enormously in tuber size, form and color. *Source: isgtw.com*

would in turn strengthen the military position of a country. The population of Europe grew very rapidly in the 18th and 19th century partly due to this new food crop, especially in Ireland. In the 19th century, Europe was confronted with the first epidemic diseases in potato and since Europe was highly dependent on potatoes for food, severe famines were the result. The first and most infamous famine took place between 1845 and 1850 in Ireland and is known as the Great Irish Famine. Of the eight million Irish people, one million died and another one million emigrated to North America. Throughout the 20th and 21st century until nowadays, the potato remained a crop of major importance.

Potato plants are susceptible to a lot of different pathogens and they can thus have a lot of different diseases. Potato pathogens include bacteria, viruses, fungi and oomycetes (Christ, 1998). As already said, *Phytophthora infestans* causes late blight diseases, which is the most devastating and economically important potato disease. This pathogen was also responsible for the Great Irish Famine. The next chapter will give a detailed overview of that deadly pathogen, *Phytophthora infestans*.

3. *Phytophthora infestans*: a pathogen that keeps surprising humanity

Phytophthora infestans is an oomycete species. Literary translated *Phytophthora infestans* means plant destroyer**.** The oomycetes are part of the stramenophiles (or heterokont) group, which is in turn part of the eukaryotic supergroup Chromalveolata (Fry, 2008). Oomycetes, sometimes called water molds or pseudofungi, have very similar characteristics as fungi, but they are not closely related to each other (Fig. 4). Both oomycetes and fungi form hyphae and both can reproduce asexually by means of sporulation and sexually by means of gametogenesis (Latijnhouwers *et al.*, 2003). These similarities between oomycetes and fungi are the result of convergent evolution to a similar niche instead of the result of common ancestry. When looking at a cellular and biochemical level, lots of differences between both groups can be detected (Latijnhouwers *et al.*, 2003). For example, the cell wall of fungi is composed of chitin, while the cell wall of oomycetes is composed of glucans, like cellulose. Besides, fungi are haploid during most of their life cycle, while oomycetes are diploid during most of their lifecycle.

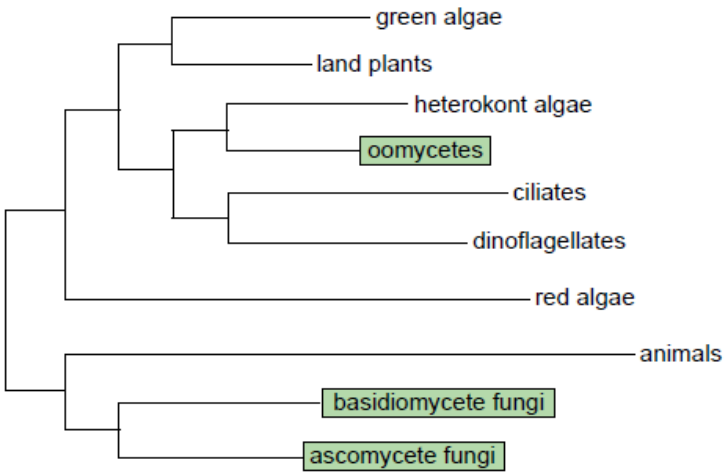

Fig. 4. Fungi and oomycetes are not closely related to each other, as can be seen in this phylogenetic tree (Latijnhouwers *et al.*, 2003).

P. infestans can survive badly as a saprophyte in the soil and it needs living material to survive: it is a near obligate plant pathogen. The pathogen is further said to be hemibiotrophic (Fry, 2008; Nowicki *et al.,* 2012). This means that the life cycle of *P. infestans* can be divided into two parts. During the first part of the life cycle the pathogen behaves like a biotroph: it grows in the plant and subtracts nutrient out of the plant cells without killing any cells. During the second part of the life cycle the pathogen behaves like a necrotroph: it kills the plant cells at the place of colonization, which is needed for the pathogen to reproduce successfully. Often, plants are damaged so much that the whole plant quickly dies. *P. infestans* has, like all oomycetes, aseptate hyphae: the vegetative growing hyphae contain multiple nuclei that are not separate by cell-wall like structures called septa (Judelson & Blanco, 2005). The nuclei are all diploid and have between 11-13 chromosomes. *P. infestans* is a heterothallic species, meaning that there are distinct sexes or mating types. These

mating types are called A1 and A2 (Fry, 2008). Sex determination in *P. infestans* is complex and not completely understood (Fry, 2008). Mating type seems to be controlled by a single gene with heterozygous individuals being the A1 type and homozygous recessive individuals being the A2 type. The alleles for the gene seem to have no simple Mendelian pattern of inheritance and the gene is located in different parts of chromosomes for different strains of *P. infestans*.

3.1 The life cycle of P. infestans: an overview

The life cycle of *P. infestans* is schematically depicted in Fig. 5. The pathogen can reproduce asexually by the formation of a sporangium. Asexual reproduction can occur in two different ways, either by asexual sporulation in which zoospores are formed in the sporangium and subsequently released, a process called zoosporogenesis or by the direct germination of the sporangium. At temperatures below 15 degrees, zoosporogenesis occurs, while at temperatures above 15 degrees direct germination occurs (Nowicki *et al.,* 2012). The pathogen can also reproduce sexually through sexual sporulation in which oospores are formed, a process called oosporogenesis. This can only take place when both mating types are present (Judelson & Blanco, 2005).

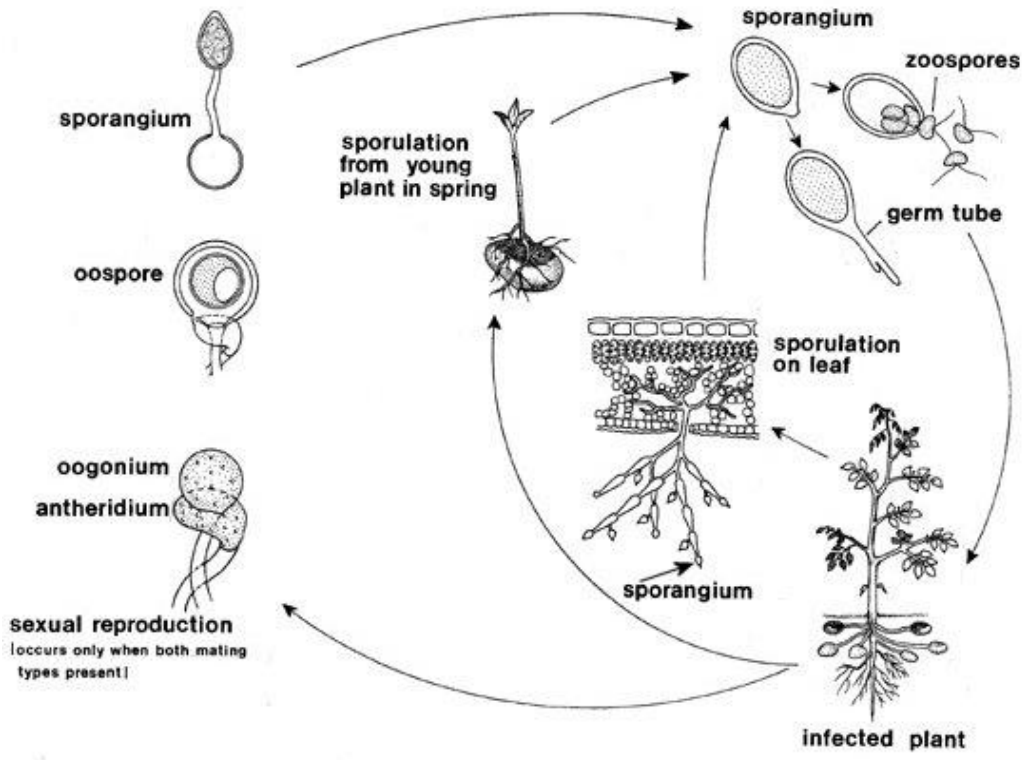

Fig. 5. The life cycle of *P. infestans*. *P. infestans* can reproduce asexually and sexually. *P. infestans* hyphae can overwinter in potato tubers. These hyphae can start sporulating again in spring, which can lead to the infection of new plant tissues. Sexual reproduction can only occur when the A1 and A2 mating type are present. It results in the production of oospores. These can germinate to form hyphae, which often quickly form sporangia from which new zoospores can be released. *Source: bioweb.uwlax.edu*

Zoospores are motile wall-less diploid spores, with two flagella on their surface (Hardham, 2005). Zoospore-mediated reproduction is contributing greatly to epidemic outbreaks of late blight in one growing season, because zoospores are often released in large amounts from a sporangium and because zoospores are motile, meaning that they can swim to a plant, when they have not landed on it. Zoospores are chemotactic, meaning that they can sense chemical gradients and use them to find a suitable place for infection. Besides, they can respond to electrical gradients. The spores can swim for approximately 60 minutes at speeds of approximately 200 µm s^{-1} and they are able to cover

distances of several centimeters (Hardham, 2005; Fry, 2008). Zoospores need much energy to stay alive and they have no cell wall that protects them so they are in direct contact with their environment and therefore need much energy to maintain cellular homeostasis (i.e. zoospores have a high risk of desiccation). Besides, zoospores need much energy for swimming with their flagella. Zoospores do therefore not live very long in the soil and contribute as a consequence very little to the survival of *P. infestans* in soils from one growing season to another (Fry, 2008). They are therefore not the cause of renewed outbreaks of late blight in fields in the next season.

Oospores are non-motile thick-walled diploid spores (Judelson & Blanco, 2005). They can survive for a long time (up to ten years) in the soil or in potato tubers: oospores are well able to survive in soil for one winter in Europe (Andrivon, 1995). They can start germinating the next growing season to form hyphae that often quickly form sporangia from which subsequently lots of zoospores can be released. These can then all infect new plant tissues. Due to the surviving capabilities of oospores, oospores contribute greatly to survival of *P. infestans* from one season to another (i.e. during the winter) and thus to the renewed outbreak of late blight in the next season. From a farmer's perspective, sexual reproduction, which results in the formation of oospores, is thus not wanted. There are more reasons why sexual reproduction is unwanted (Nowicki *et al.,* 2012). Sexual reproduction results in new combinations of genetic information and thus new genetic diversity. These new combinations might contribute to the success of the pathogen. Moreover, beneficial mutations that have arisen in different asexually reproducing lineages can come together, resulting in a more virulent pathogen that displaces the less adapted populations. Bad mutations can be filtered out due to sexual reproduction. These new genetic types might thus not respond as wanted to the currently used methods to control late blight disease in agriculture. In asexually reproducing populations of *P. infestans* new genetic variation and changes in the genetic composition of populations can also occur, though at a much lower speed than in sexual reproducing populations.

A very important characteristic of *P. infestans* is that it has a high degree of plasticity (Haas *et al.*, 2009). The organism is able to evolve quickly and can therefore also adapt itself very quickly to changing environments and selection pressures, thereby keeping its pathogenicity high. The exact mechanism that underlies the plasticity of *P. infestans* will be discussed later.

Until recently *P. infestans* propagated itself only asexually in most parts of the world, but now it also starts reproducing sexually in more and more parts of the world (Judelson & Blanco, 2005). This results in late blight management strategies not working well anymore. To understand why these problems are nowadays arising, the next paragraph discusses the origin and spread of *P. infestans* all over the world.

3.1 The origin of P. infestans and its spread over the world

Until very recently, it was highly debated were the genus *Phytophthora* and in particular *P. infestans* originated. Some believed it originated in Peruvian Andes, because potato species richness was very high there and many endemic species live there (Gomez-Alpizar *et al.*, 2007; Goss *et al.*, 2014). It was thought that co-evolution between potato species and *Phytophthora* species resulted in the origin of *P. infestans*. Others believed it originated in the Toluca Valley in Central Mexico, because it was until recently the only place where sexual reproduction occurred and there was a lot of nuclear genetic variation at that place. Gomez-Alpizar *et al.* (2007) studied the variation at several nuclear and mitochondrial loci and used this information to make phylogenetic trees to elucidate the origin of *P. infestans*. They concluded that there are two mitochondrial haplotypes of *P. infestans* present in the South American Andes, while there is only one haplotype present in Central Mexico. The study therefore concluded that *P. infestans* must have originated in the Andes, probably in Peru and that *P. infestans* had migrated some later to Central Mexico. Subsequently, something must have changed in the genome of *P. infestans* resulting in the evolution of mating types and oosporogenesis in Central Mexico. Goss *et al.* (2014) have looked critically at the study of Gomez-Alpizar *et al.* and they disagreed with the conclusions: Gomez-Alpizar *et al.* had chosen a wrong species to root the

phylogenetic tree. Goss *et al.* therefore extended the study of Gomez-Alpizar *et al.* and concluded on the basis of phylogenetic analyses that *P. infestans* must have originated in Central Mexico and that some later it migrated to the Andes. In the Andes, *Phytophthora infestans* underwent adaptive radiations, resulting in many varieties and populations and probably even in new species. The presence of two different mitochondrial haplotypes in the Andes was probably the result of human interference. Most people therefore agree nowadays that *P. infestans* originated in the Toluca Valley in Central Mexico.

Probably different lineages (at least two) of *P. infestans* were carried by humans from Central Mexico to the USA. Based on DNA sequences of 19th century *P. infestans* strains, it can be concluded that in the 1840s one or multiple introductions of *P. infestans* occurred in Europa from the USA (Martin *et al.*, 2013). One or several of these lineages caused late blight epidemics, with the Great Irish Famine being the most infamous. One genetic type or lineage, designed US-1, was not present by that time, but started dominating Europe some later, thereby replacing older lineages. During the 19th century this lineage dominated for several decades, but it was replaced at some moment by the US-8 lineage that continues dominating till today in many parts of the world (Peters *et al.*, 2014). Nevertheless, this lineage is dominating less and less in many parts of the world. As said earlier, until recently *P. infestans* propagated asexually in most parts of the world expect for Central Mexico. All populations were namely of the A1 mating type. However, in the 1970s the A2 mating type arrived in Europe by ship from Central Mexico. The ship did also contain many new genetic types of *P. infestans* and after the 1970s, oosporogenesis was observed in Europe and new and badly manageable strains of *P. infestans* did arise (Fry, 2008). From Europa the A2 mating type spread to all other continents, but interestingly not to North America. Around 1990, another distinct migration of the A2 mating type occurred from Central Mexico to North America so oosporogenesis also started to occur there (Fry, 2008).

Problems with late blight epidemics are increasing in Europe. The genetic composition of the Dutch *P. infestans* population changed dramatically during the 10-year period 2000-2009 (Li *et al.*, 2012). Several strains with A2 mating types have spread across the Netherlands and sexual reproduction occurred more. Nevertheless, asexual reproduction remained an important mechanism for successful genetic types to spread quickly. The *P. infestans* population in the Northeastern part of the Netherlands had a slightly different genetic composition then the populations in the other parts of the country: sexual reproduction and late blight related problems played a bigger role in that area. This might be caused by the greater tolerance of people towards late blight in the end of the season or through the shorter crop rotation times in that area.

In Canada population composition is changing rapidly, but this is mainly due to migrations of clonally propagating lineages and subsequently adaptation to new environments (Peters *et al.*, 2014). Some new genetic types of *P. infestans* have arisen in Canada due to these processes. Due to the higher mobility of people and globalization, asexual reproducing strains of *P. infestans* can be spread to more and more parts of the world at an ever higher speed, a process that stimulates the genetic diversification of *P. infestans* strains due to adaptation of the pathogen to new environments. This also promotes the problems with the control of late blight, though nevertheless sexual reproduction in *P. infestans* is nowadays the main problem. Asexual strains of *P. infestans* that had immigrated in new areas in North America can be more aggressive than the older lineages that are already present in an area. Miller & Johnson (2014) showed that amongst other the more recently arrived US-1 lineage was generally more aggressive than the earlier arrived US-8 lineage: the US-1 lineage had a larger temperature range in which it was able to infect potato plants. Oosporogenesis-related problems seem to just start seriously affecting North America: in one area in Canada, both A1 and A2 mating types were found and *P. infestans* isolates collected from tomato in 2010 showed evidence for sexual reproduction (Peters *et al.*, 2014).

3.3 A short recap: why are late blight problems increasing?

Problems with late blight were always large due to the high potential of *P. infestans* to adapt to new circumstances, but problems are increasing in recent decades: this can be partly explained by the faster origin of new asexual strains, but it is mainly caused by the emerge of sexual reproduction in many parts of the world. The next two chapters will discuss zoosporogenesis and oosporogenesis is more detail.

4. Zoosporogenesis: from sporangium to new individuals

A very important first thing that happens during zoosporogenesis is the formation of multinucleate sporangia at the tips of vegetative growing hyphae (Judelson & Blanco, 2005). These sporangia are located on special structures called sporangiophores, which are stalk-like structures that are often branched (Fig. 6). Sporangiophores often contain multiple sporangia, one per branch. The metabolism and physiology of the hyphal tips changes dramatically when they develop into a sporangium. At maturity, the cytoplasm of the sporangium is separated from the rest of the hyphae due to the formation of a basal septum and typically contains about 20-30 pyriform (pear shaped) nuclei, most of which are located near the sporangial wall. They are kept in place by microtubules (Hardham, 2005). Besides, a mature sporangium contains many vesicles. It is not completely clear what triggers the formation of sporangia, but nutrient limitation, high humidity, high oxygen and low carbon dioxide concentrations probably play a role (Judelson & Blanco, 2005). These are sensed by receptors already present in vegetative growing hyphae. Mature sporangia can detach from the hyphae and can be transported to another place.

Sporangia can remain in a state of dormancy for some time, but not for very long since sporangia are metabolically active to prevent themselves from desiccation (Judelson & Blanco, 2005). After some time, the sporangium can either start germinating directly or start with the formation of zoospores. *P. infestans* prefers to sporulate during dark periods, so the amount of light might somehow trigger zoosporogenesis (Nowicki *et al.,* 2012). During zoospore formation, small electron-dense vesicles and Golgi vesicles fuse to form membranes that separate the nuclei in the sporangium from each other (Hardham, 2005). Two flagella are subsequently placed at the cell membrane of the zoospore in being. The anterior flagellum is the shortest and bears so-called mastigonemes on it. These are straw-like “hairs”, the defining character of the Stramenophiles. Sinusoidal waves are propagated along the anterior flagellum, which pulls the zoospore forward. The posterior flagellum is the longest and does not contain mastigonemes. It is used as for steering the zoospore. So-called large dorsal and ventral vesicles that are already present in a mature sporangium become localized at respectively the dorsal and ventral part of the zoospore during zoospore formation (Hardham, 2005). These two types of vesicles differ in content. Besides, lots of mitochondria are transported into the zoospores.

Some aspects of the genetic architecture of zoosporogenesis in *P. infestans* are known. The NIF gene family is a family of phosphatase-encoding genes that are highly upregulated during zoosporogenesis (Tani & Judelson, 2006). It is thought that these genes have a role in initiating zoosporogenesis. NIFs probably change the phosphorylation state of RNA polymerases or interact with other regulatory proteins. The control of transcription of *PiNIFC3*, a NIF gene, by temperature has been thoroughly studied (Tani & Judelson, 2006). Cold temperatures make cell membranes more rigid. Increased membrane rigidity is probably sensed by phospholipase C and inositol triphosphate. These proteins are present in plasma membranes and mediate Ca^{2+} entry in the cell. Increased membrane rigidity lowers the Ca^{2+} concentrations in the cell. Lower Ca^{2+} concentrations in the cell might then change intracellular signaling pathways. These changes (or maybe Ca^{2+} directly) interact in an unknown way with a seven-nucleotide motif called the cold box that is located upstream of the *PiNIFC3* gene. The gene expression of *PiNIFC3* is then upregulated. Since cold boxes and found in several other NIF genes and in several other zoosporogenesis-induced genes, the above described

pathway is probably applicable to lots of genes and explains how cold triggers zoosporogenesis. These changes in gene expression can take place within minutes after cold exposure.

Another gene that is strongly upregulated during zoosporogenesis is the Cdc14 phosphatase gene (Ah-Fong & Judelson, 2011). This gene is not expressed in vegetative growing hyphae, but only during zoosporogenesis. In most eukaryotic species, Cdc14 regulates the cell cycle by regulated mitosis. This is definitely not the function in oomycetes, since sporangia do not undergo mitosis. In sporangia, Cdc14 proteins accumulate in nuclei during early zoosporogenesis, which can be shown by fusing Cdc14 to the reporter protein GFP. Some later in zoosporogenesis, Cdc14 proteins can be found accumulating in basal bodies, which are located at the base of the flagella of the zoospore. Cdc14 interacts with microtubules and can bind to them. Overexpression of the Cdc14 gene by placing the gene behind a strong promotor resulted in several defects during zoosporogenesis: the formation of membranes that separate the nuclei in a sporangium from each other went wrong. Silencing of the Cdc14 gene resulted in failures to form sporangia. Cdc14 is thus very important for zoosporogenesis to occur in the right way, probably by interacting with microtubules.

When the zoospores are formed, hydrostatic pressure is build up in the sporangium (Hardham, 2005). Solutes are accumulated in the fluid between the zoospores, thereby increasing the osmotic value of the fluid. To prevent desiccation, zoospores have to synthesize osmolytes as well: they synthesize proline. When hydrostatic pressure is high enough, the tip of the sporangium breaks open and the zoospores are released. Proline is then quickly degraded in the zoospore to prevent the explosion of the zoospore: proline biosynthetic genes are upregulated.

When a zoospore has reached the surface of a plant (either the sprout or the tuber) after swimming to it, it orients so that the ventral vesicles face the plant surface. It then starts encysting: the zoospore becomes sessile (Hardham, 2005). Several vesicles then fuse with the plasma membrane of the zoospore, thereby probably changing the properties of it. In two minutes, the content of the dorsal vesicles is secreted to form a protective mucus-like coating that surrounds the zoospore. Besides, the content of the ventral vesicles is secreted to form an adhesive medium between the plant surface and the zoospore and within 5-10 minutes, the zoospore develops a cell wall. After 20-30 minutes, a germ tube grows from the cyst. It grows along the surface of the plant or enters the plant via stomata, lenticels or wounds. Often, an appressorium is formed from the germ tube. This is a swollen and flat organ that presses on the plant cell wall. This makes it easier for the hyphae to penetrate the cell wall during an infection. A large scale infection can be established in a few hours and the asexual life cycle can start all over again (Judelson & Blanco, 2005). The infection process will be discussed in detail later.

During the asexual life cycle of *P. infestans* overall gene expression changes dramatically (Fig. 6L,K). DNA microarray studies and qPCR reactions revealed that more than fifty percent of the genes of *P. infestans* changed in expression during the asexual life cycle, with ten percent being expressed in only one life-stage (Judelson *et al.*, 2008). Among the genes with strong changes in expression were a lot of putative regulatory genes, but also genes involved in energy metabolism and pathogenicity. The genes with a changed gene expression are now studied in more detail to reveal their exact functioning and effects in the asexual life cycle. The effects of changed gene expression are namely not directly deducible from DNA microarray essays. For example, some genes involved in glycolysis were upregulated in zoospores, while others were downregulated.

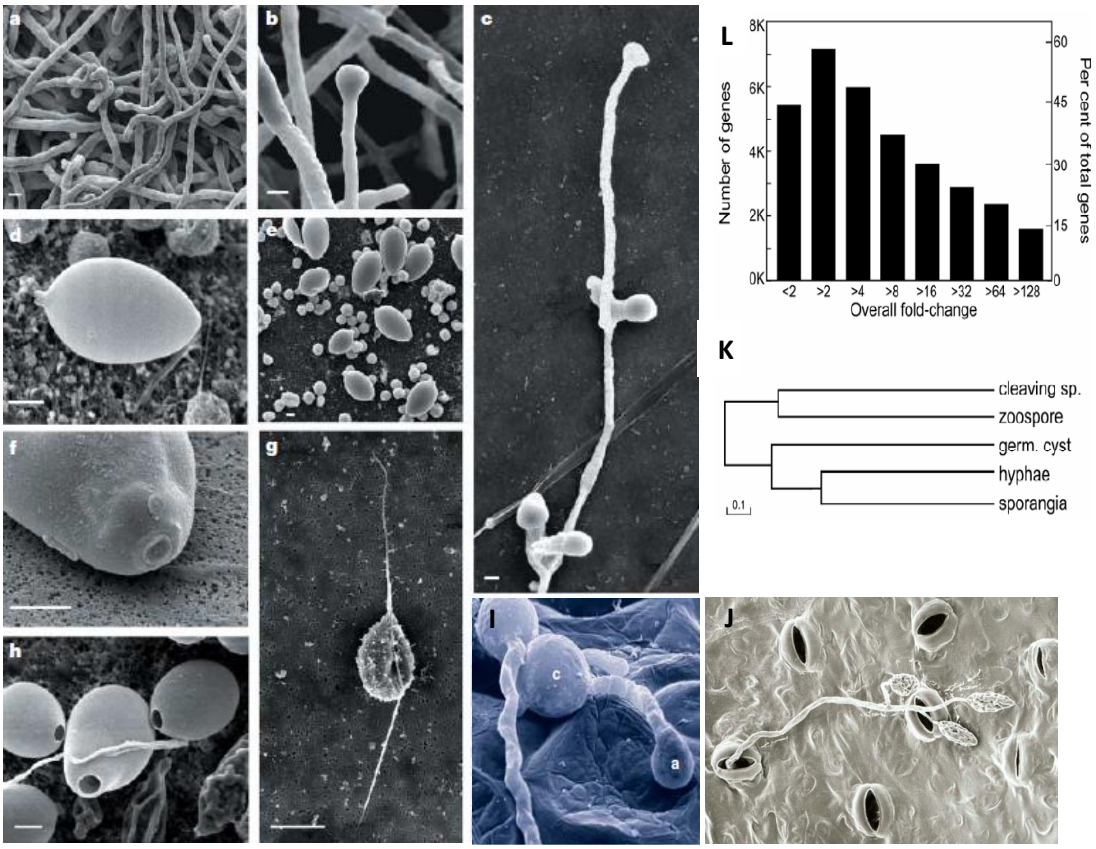

Fig. 6. An overview of zoosporogenesis. **A**: Vegetative, non-sporulating hyphae. **B**: The swollen tip of a sporangiophore. This is the initial of a sporangium. **C**: A sporangiophore containing four mature sporangia on lateral branches and a terminal sporangiophore initial. **D**: An ungerminated sporangium. **E**: A mixture of sporangia and zoospores. **F**: The tip of a sporangium, showing the opening through which zoospores are released. It is closed with a plug on this photograph. **G**: A zoospore with its two flagella. Mastigonemes can be seen on the upper flagellum. **H**: sporangia after releasing zoospores. The opening is unplugged on this photograph. **I**: The development of an appressorium (a) from a cyst (c). **J**: A sporangiophore containing sporangia has grown through the opening of a stomata (Judelson & Blanco, 2005). **L**: Diagram showing that approximately 60% of the genes of *P. infestans* changes more than two-fold in expression during asexual reproduction. **K**: Tree showing the amount of similarity in gene expression between different developmental stages. Note that mature sporangia and sporangia that undergo zoosporogenesis (cleaving sporangia) are very different. Lots of genes change in expression at the onset of zoosporogenesis (Judelson *et al.*, 2008).

Oosporogenesis: the cause of big problems

The A1 and A2 mating type of *P. infestans* secrete mating-type specific hormones and these can be sensed by other *P. infestans* individuals (Judelson & Blanco, 2005; Prakob & Judelson, 2007; Fry, 2008). When two different mating types sense each other's hormones, they develop gametangia at the hyphal tips (Fig. 7). Male gametangia are called antheridia, while female gametangia are called oogonia. Each mating type is capable to form both antheridia and oogonia. *P. infestans* individuals can therefore fertilize themselves, though mostly cross-fertilization occurs, probably due to some yet unknown mechanism. In the gametangia meiosis occurs, which produces haploid gametes. The antheridia and oogonia grow towards each other and when both are in physical contact, nuclei migrate from the antheridia to the oogonia and these subsequently fuse with female gametes. A thick multilayered wall develops around the diploid nucleus, consisting of amongst others glucose polymers. Besides, the cytoplasm of the oospore in being is filled with energy rich molecules. When these processes are finished, the oospore is ready to be released and is able to infect new plant tissues.

DNA microarray studies and qPCR reactions revealed that 87 of the 15.644 genes of *P. infestans* were expressed more than ten-fold during oosporogenesis (Prakob & Judelson, 2007). The

role of 44 of these genes remains entirely unknown, but most of genes with known functions were regulatory genes or involved in metabolism. For example, genes that were probably involved in making the cell wall of the oospores and genes that were involved in lipid synthesis were highly upregulated. Ten genes were upregulated during both sexual and asexual reproduction, suggesting some crosstalk between both forms of reproduction. Both sexual and asexual reproduction seems to be stimulated by nutrient limitation. Nevertheless, in zones were sexual reproduction dominates; asexual reproduction is suppressed, meaning that both pathways are not completely regulated in the same way. The mechanism that regulates this antagonistic interaction and the exact molecular and genetic differences between asexual and sexual reproduction remain to be elucidated.

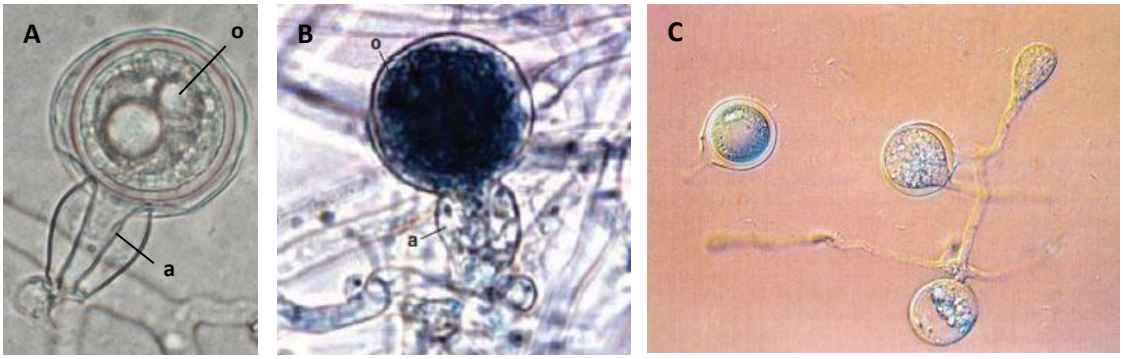

Fig. 7. A, B: An oogonium containing an oospore, denoted with (o). The antheridium is denoted with (a). **C**: Germinating oospores. The right oospores germinated to form short hyphae that have formed sporangia. The left oospore has just started germinating (Judelson & Blanco, 2005).

6. The infection phase: an ongoing co-evolution between host and pathogen

Most potato cultivars are susceptible to late blight, because their immune systems are not effective enough against *P. infestans*. Plants possess physical defenses against pathogens that function as a first line of defense. For example, the cuticle prevents direct entry of pathogens in the plant. Moreover, plants constitutively produce toxic compounds as a second line of defense. The innate immune system of plants forms a third line of defense and can be divided in two branches (Jones & Dangl, 2006).

6.1 PAMP-triggered immunity

Pathogens possess conserved features that evolve slowly and that are as a consequence conserved within a group of species (Jones & Dangl, 2006). An example is chitin, the component of fungal cell walls, which characterizes fungi. These features, called pathogen-associated molecular patterns (PAMP), are used by plants to recognize broad groups of pathogens. Pathogen-recognition receptors (PRRs) that are present in the plant cell membrane bind to these PAMPs, which result in the activation of intracellular signaling pathways that often result in changes in the transcription of genes in the plant cell. Plant cells can then start secreting hydrolytic enzymes, like antimicrobial peptides, secrete phytoalexins or deposit lignin at the place of a pathogen infection as a barrier for the further spread of the pathogen. Another very common response to pathogens is the hypersensitive response (HR) in which the cells surrounding the place of infections rapidly die to prevent the further spread of the pathogen (Jones & Dangl, 2006). Often the cells at the place of infection secrete signaling compounds, like methyl salicylic acid and jasmonic acid, to “warn” other parts of the plant: these compounds activate pathogenesis-related (PR) genes that encode different kinds of antimicrobial peptides and enzymes in plant cells in every part of the plant. This leads to a higher resistance in all

parts of the plant to a secondary infection with the pathogen, a phenomenon called systemic acquired resistance (SAR).

A PAMP that characterizes the *Phytophthora* genus is the peptide fragment Pep-13, which is present in all *Phytophthora* species (Brunner *et al.*, 2002). This fragment is part of an abundant cell wall glycoprotein that functions as a Ca^{2+}-dependent transglutaminase. Transglutaminases are involved in protein crosslinking. Mutations in the Pep-13 region seem to result in not functional transglutaminases and since proper functioning of these enzymes is necessary for an individual to survive, transglutaminases are evolutionary conserved. It was investigated how Pep-13 triggered an immune response in potato by using transgenic plants that were unable to react to jasmonic acid (JA) and salicylic acid (SA) (Halim *et al.*, 2009). It was thought that these two plant hormones were involved in the hypersensitivity response induced by Pep-13. SA accumulated in both JA-sensitive and JA-insensitive plants, suggesting that SA accumulates independently of JA. Besides, these plants were able to form H_2O_2 and establish HR. Nevertheless, in these JA-insensitive plants much lower concentrations of H_2O_2 were found and HR occurred much less than in JA-sensitive plants, suggesting SA can induce HR and that this process is stimulated by JA (Fig. 8). Solely JA or SA controlled the gene expression of some defense genes, while both JA and SA controlled others.

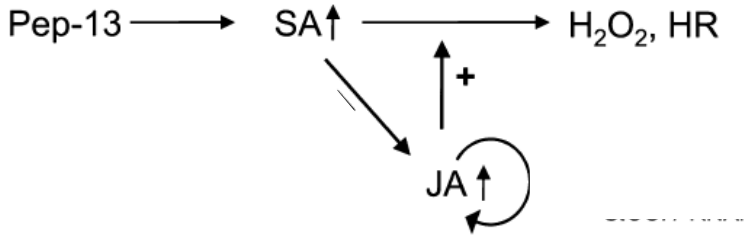

Fig. 8. The proposed mechanism for Pep-13 triggered HR. SA mediates Pep-13 triggered HR. JA stimulates Pep-13 triggered HR (Halim *et al.*, 2009).

6.2 Effector-triggered immunity

Although all potato cultivars recognize Pep-13, most of them are still highly susceptible to late blight. Successful pathogens produce molecules called effectors that are used to support the pathogen in successfully colonizing a plant (Nowicki *et al.,* 2012). Effectors can work either extracellular or intracellular. Most effectors are secreted by haustoria: finger-like feeding structures that form from the vegetative growing hyphae of *P. infestans* after it has entered a plant (Fig. 9) (Judelson & Blanco, 2005). Haustoria are located inside the cell wall of a plant cell, but they do not penetrate into the cytoplasm of the cell. They surround the protoplast and are used to subtract nutrients from the plant cells without killing them. Extracellular acting effectors can function as hydrolytic enzymes that degrade plant cell walls, thereby generating space for the hyphae to grow further in the plant tissue. Besides, some effectors function as proteases or as other protective molecules that degrade plant proteins, such as plant-derived hydrolytic enzymes and antimicrobial peptides. Intracellular acting effectors bring about changes in the physiology and metabolism of the cell in the advance of the pathogen, as will be discussed later.

The formation of haustoria has been studied in some molecular detail (Avrova *et al.*, 2008). In germinating cysts and appressoria the *pihmp1* gene is upregulated. This gene encoded a transmembrane protein and silencing of the gene resulted in the loss of pathogenicity for *P. infestans*, because it was then unable to form haustoria. Using the red fluorescent protein, it was shown that in germinating hyphae, the concentrations of *pihmp1* proteins were high at certain locations in the hyphae. These locations did later develop into haustoria, suggesting that *pihmp1* "marks" the places that have to become haustoria. In mature hyphae, the *pihmp1* protein was solely present in the haustorial membrane. The function of the protein is unknown, but it is possibly a structural protein that stabilizes the cell membrane and perhaps the cell wall of hyphae when the hyphae grow further between the plant cells. Silencing of the *pihmp1* gene made *P. infestans* unable to penetrate in the plant.

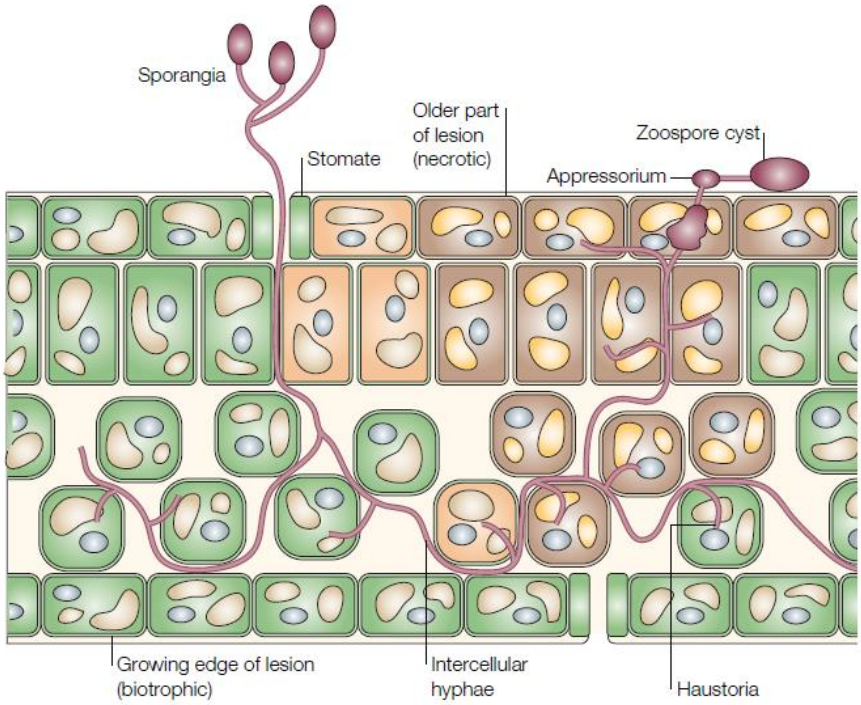

Fig. 9. The course of infection by *P. infestans*. Haustoria are schematically depicted: they penetrate plant cell walls, but not the cell membrane. Brown cells represent dead cells, while orange cells represent dying cells. Lesions can quickly enlarge (Judelson & Blanco, 2005).

Extracellular effectors can be sensed by PRRs and this can subsequently induce PAMP-triggered immunity. Intracellular effectors can nevertheless not be sensed by PRRs: how can a plant then sense these molecules (Fig. 10)? Plants can sense them, because they have evolved so-called Resistance (R) proteins, which are encoded by Resistance (R) genes (Fry, 2008). The most common types of R proteins are the nucleotide binding site-leucine rich repeat receptors (NBS-LRRs). When an intracellular effector and some cytoplasmic plant molecule interact, an R protein senses this: it subsequently becomes activated and induces cellular gene expression to change. All the things already noted in the previous paragraph can happen: from the HR to the SAR. This is called effector-triggered immunity (ETI) and it is a much stronger anti-pathogenic response than PAMP-triggered immunity (Jones & Dangl, 2006). Most R proteins only recognize one type of effector, but some can nevertheless even recognize effectors from different *Phytophthora* species (Fry, 2008). Following the gene-for-gene hypothesis, all effectors that contribute to the virulence of the pathogen have to be recognized by R proteins to be immune against it.

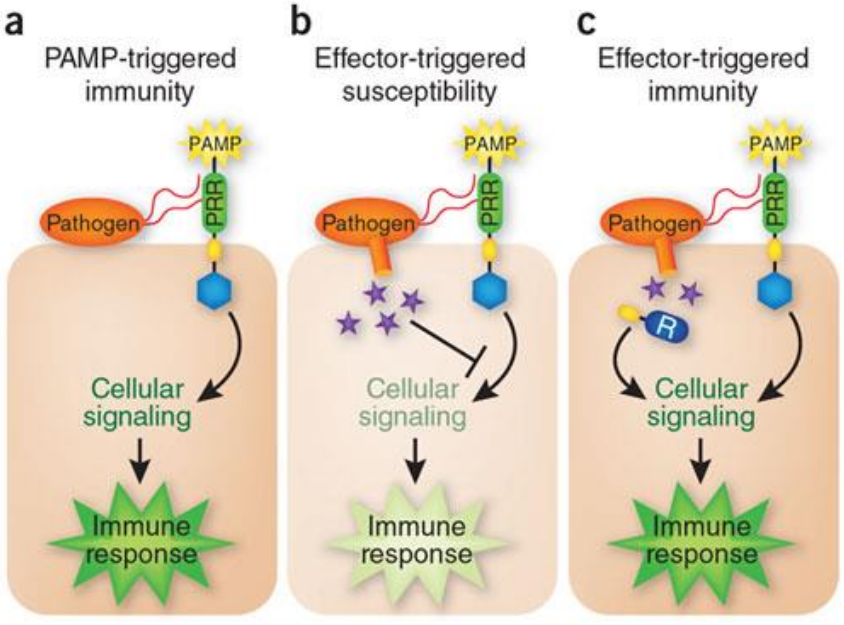

Fig. 10. Plants can recognize PAMPs using PRRs and that can trigger an immune response (**A**). To circumvent this immune response, pathogens have evolved effectors that are secreted in the cytoplasm of plant cells (**B**). These cannot be recognized by PRRs, making plants susceptible for the pathogen. Intracellular effector might block or interfere with PAMP-triggered immunity. This is known as effector-triggered susceptibility. As an answer to this problem, plants have evolved R genes that become activated when the effector and a certain cytoplasmic molecule interact (**C**). This is known as effector-triggered immunity. *Source: jonlieffmd.com*

Effectors are often species-specific and can therefore be used by plants to determine the exact species with which they are confronted. The genes that encode effectors are evolving fast, thereby giving the pathogen many opportunities to "break" the existing resistances in plants. Through mutations, pathogens acquire effectors that are no longer recognized by the plants, but plants can in turn acquire R genes via mutations that do recognize these new effectors (Nowicki *et al.,* 2012). There is an ongoing co-evolution between plant and pathogen. As a consequence, the genome of *P. infestans* contains many effector genes and the genome of potato plants contains many R genes.

The genome sequence of *P. infestans* has provided an explanation for the enormous ability of this organism to break resistances (Haas *et al.*, 2009). The genome contains many regions that are highly conserved and gene dense: many genes that encode basic cellular functions are located there. The regions between these blocks are highly variable, contain many repetitive DNA fragments and transposons and are not gene dense: most effector genes are located in these regions. Effector gene families were rapidly diversifying, creating new genes and alleles. Besides, the regions contained many inactive pseudogenes, underlining that effectors do indeed undergo rapid evolution.

Two types of intracellular effector gene families exist: the RXLR gene family and the CRN gene family (Nowicki *et al.,* 2012). The defining character of the RXLR family is that all the effectors have the conserved amino acid sequence RXLR (Arg-X-Leu-Arg with X being a variable amino acid) at their N-terminal (Whisson *et al.*, 2007). Some of these effectors have an EER motif (Glu-Glu-Arg) at less than 25 residues downstream the RXLR motif and these specific effectors are therefore called RXLR-EER effectors. The C-terminal of the RXLR effectors is highly variable: it determines the exact functioning of the effector and can, as stated, evolve rapidly. One of such effectors is the avirulence protein 3a (Avr3a): R proteins can recognize them once they are in the cytoplasm and respond with the HR. The ability of Avr3a to trigger HR was used to see if the RXLR-EER motif functions as a translocation signal into plants cells (Fig. 11) (Whisson *et al.*, 2007). When either the RXLR motif was substituted with arginine residues or when this was done with the EER motif or with both no HR occurred. This suggests that the motifs play indeed a role as a translocation signal, since HR occurs only when Avr3a is inside the cell. Using the red fluorescent protein as a reporter molecule, it was shown that RXLR-EER effectors were secreted by haustoria into the so-called extrahaustorial matrix, the small space between haustoria and protoplast. The RXLR-EER motif was not involved in secretion by the haustoria, because the effectors with alanine substitutes were also secreted: the motif was solely involved in cellular uptake (Whisson *et al.*, 2007). RXLR-EER effectors were able to enter plant cells when they were added to them without the pathogen present, meaning that they use receptors

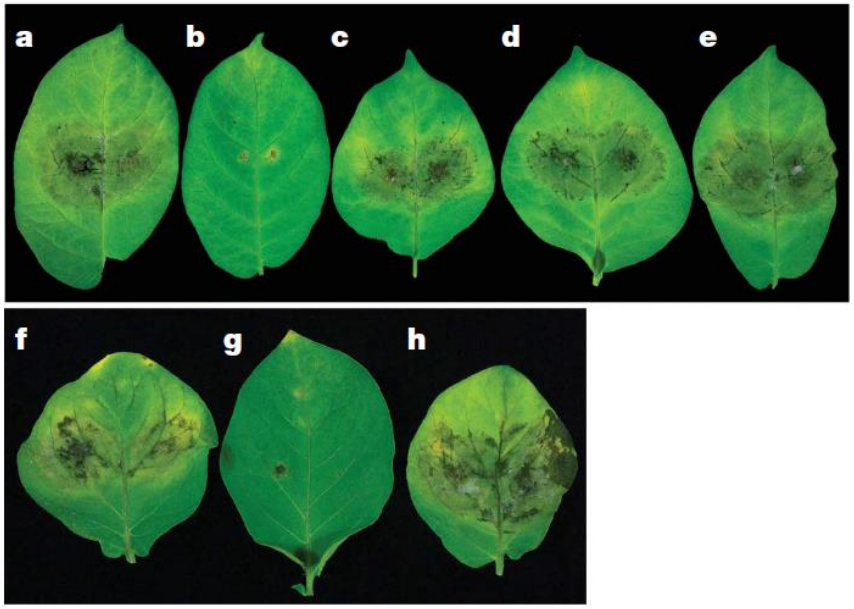

Fig. 11. Replacement of RXLR-EER motifs with alanine residues, singly or in combination, or with amino acids KMIK-DDK, prevents delivery of Avr3a into host cells. The leaves of the potato cultivar Pentland Ace were infected with: **A & F**: a *P. infestans* strain that does not have Avr3a, but avr3a. This is an allele that cannot be recognized by the plant. Leaves are infected. **B & G**: a strain that has Avr3a. HR occurs and the leaves are not successfully infected. **C**: a strain that has the RXLR motif replaced by alanine residues. The leaf is infected. **D**: a strain that has the EER motif replaced. The leaf is infected. **E**: a strain that has both the RXLR and EER motifs replaced. The leaf is infected. **H**: a strain that has the RXLR and EER motif replaced with the amino acids KMIK-DDK, as a sort of control. The leaf is infected (Whisson *et al.*, 2007) .

already present on plant cells rather than pathogen encoded machinery or something else from the pathogen. It is thought that RXLR-EER motif binds to the PI-3-P membrane receptor that is present on plant cells and that the effector is then subsequently taken up in the cell through endocytosis (Kale & Tyler, 2011). Later research did show that some RXLR-EER effectors were secreted by vegetative growing hyphae into the apoplast and these were also taken up in the cells through endocytosis (Fig. 12).

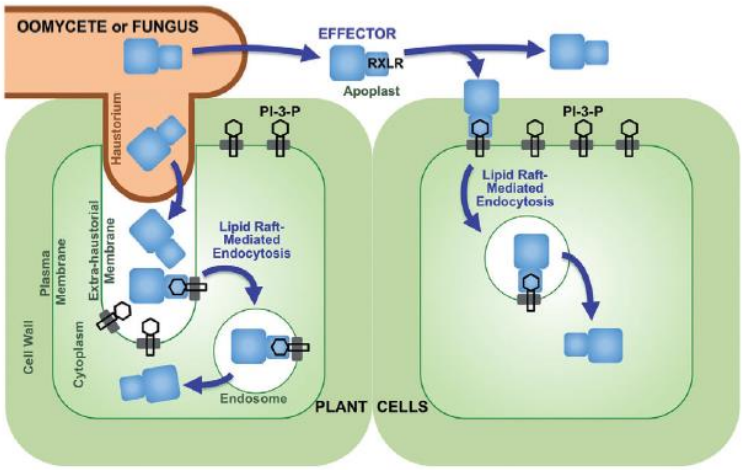

Fig. 12. Current model for the entry of RXLR-effectors in plant cells. RXLR-effectors are secreted by haustoria in the extrahaustorial matrix or by hyphae in the apoplast. They enter cells via endocytosis mediated by PI-3-P receptors. It is unknown how the effectors exit the endosomes (Kale & Tyler, 2011).

As stated earlier, Avr3a is an intracellular effector and it is very important for causing the pathogenicity of *P. infestans* (Bos *et al.*, 2010). Silencing of the Avr3a gene resulted in significantly lowered pathogenicity. Research has tried to elucidate the exact functioning of Avr3a. An extracellular working effector secreted by *P. infestans* called INF-1 triggers cell death in plants as part of the effector-triggered immunity: it triggers the hypersensitive response (HR), because it is recognized by the plant. *P. infestans* does not want the HR to occur during the biotrophic phase of the life cycle, since it then needs living cells. An ubiquitinating enzyme called CMPG1 is in that situation present at low concentrations, because it is constantly degraded by 26S proteasomes. Avr3a can interact with CMPG1 and stabilize it by modifying a specific domain of CMPG1 called the U-box in some unknown way (Bos *et al.*, 2010). This results in a changed activity of CMPG1 and prevents the HR from occurring. Avr3a thus functions as a repressor of the HR. During the necrotrophic phase of the life cycle, *P. infestans* does decrease the Avr3a transcripts in abundance, while INF-1 transcripts are increasing, thereby stimulating HR-mediated cell death that is now advantageous for the pathogen: the pathogen then thus makes use of the HR to kill cells.

The CRN (Crinkler and Necrosis) effectors are defined by having a conserved LXLFLAK motif (Leu-X-Leu-Phe-Leu-Ala-Lys with X being a variable amino acid) at their N-terminal (Schornack *et al.,* 2010). This motif is used to translocate the effectors to the nucleus: the motif binds to a receptor in the nuclear membrane, importin-α, that mediates the transport of CRNs across the membrane (Schornack *et al.,* 2010). The C-termini of these effectors determine the exact function of the molecule and are therefore highly variable. CRNs change the physiology of the cell to the advantage of the pathogen by disturbing processes in the nucleus in ways that are not yet known. CRNs can be recognized by R proteins, which trigger HR-mediated cell death.

6.3 RNA silencing

Another, often forgotten, part of the immune system of plants makes use of a phenomenon called RNA silencing. It is known since some time that plants synthesize small RNA fragments that are complementary and can bind to the RNA molecules synthesized by pathogenic viruses and bacteria, thereby making it impossible for translation of these RNA molecules to occur. This is RNA silencing. To protect themselves against RNA silencing, viruses and bacteria synthesize effectors that suppress

RNA silencing. A study performed by Qiao *et al.* (2013) showed that plants do also use RNA silencing to protect themselves against eukaryotic pathogens. The genome of *Phytophthora sojae* contained effectors that suppress RNA silencing and a homologue of one of these effectors has been found in the genome of *Phytophthora infestans.* The effectors were inhibiting the synthesis of small RNAs, which promoted the chances of successful colonization of *P. sojae*.

7. Management strategies: keeping potatoes healthy

Knowledge about the interactions between potato plant and *P. infestans* is used to design management strategies that tackle the problem of late blight. To design good management strategies, monitoring studies are performed to see how late blight disease spreads on and between farms. A study performed in the Dutch province of Flevoland from 1994 to 1996 showed that refuse piles (waste containing potato that are not used anymore) were major sources from which *P. infestans* spores could spread to potato plants growing in the field (Zwankhuizen *et al.*, 1998). *P. infestans* hyphae can survive during the winter in these potatoes and start sporulating again in spring. Infected seed potatoes and oospores were other inocula. Organic farms, at which no synthetic fungicides are used to control late blight, were major sources from which *P. infestans* did spread to surrounding traditional farms. Organic farms were more susceptible to late blight than traditional farms. Besides, the study confirmed that wind is important in explaining disease gradients: fields that were located downwind refuse piles were often infected with late blight.

Management strategies work by disturbing the life cycle of *P. infestans*: they can prevent the formation or spread of spores and disrupt normal development of these spores. Some strategies are aimed at killing the *P. infestans* individuals present, but most strategies are preventive: they are aimed at preventing late blight from occurring (HPA, 2014). Since *P. infestans* populations have a high genetic diversity and can diversify rapidly, the most efficient control of late blight is the use of multiple strategies at the same time (Nowicki *et al.,* 2010). The use of multiple strategies increases the chances that all different *P. infestans* strains will be affected and it diminishes the chances that resistant strains will develop. In the next paragraphs, different late blight control strategies will be discussed and after that an integrative approach to late blight will be presented, using the Netherlands as an example.

7.1 Fungicides

Today, the most used strategy to control late blight in traditional agriculture is the application of fungicides to the potato plants (Nowicki *et al.,* 2010). In organic agriculture, fungicides are not used, though organic agriculture does sometimes make use of nature-derived compounds, as is discussed later. Fungicides can be classified as either specific or non-specific, depending on whether they interfere with one or more processes in the pathogen (Gisi & Sierotzki, 2008). Besides, fungicides can be classified as either protectant or systemic: protectant fungicides are not taken up by the plant and thus provide only protection at the place of spraying, while systemic fungicides are taken up by the plant and transported to other parts of the plant, thereby protecting more parts of the plant. Fungicides should be sprayed preventively on the plants: once a plant is diseased with late blight, it cannot be cured anymore and it will probably infect other plants.

In the 1930s fungicides were used for the first time(Gisi & Sierotzki, 2008). These fungicides were often protectant and non-specific, meaning that they interact with more than one process in the plant. A substance that was widely used is called the Bordeaux mixture. It consists of amongst others copper ions that bind to different kinds of enzymes in spores, thereby preventing germination. The mixture is nowadays banned in most countries, since it caused toxic copper ions to accumulate in the environment and in the food of humans.

In the last decades of the 20th century a series of new often systemic and specific fungicides were developed (Gisi & Sierotzki, 2008). The mechanism by which these fungicides work does not

cause environmental and human health problems. The QoI fungicides, which block the electron transport chain at cytochrome b in the mitochondria of *P. infestans*, and the phenylamide fungicides, which inhibit RNA polymerase I, are often used. Since these fungicides have only one target in *P. infestans*, the pathogen can often become resistant to them very fast. Resistance to phenylamides is a one-locus trait, meaning that one or at maximum of a few mutations at that locus can cause *P. infestans* to become resistant (Gisi & Cohen, 1996). Metalaxyl resistance, an often used phenylamide fungicide, occurred just two years after it was used for the first time and for that reason the Netherlands has even banned its use for a while.

As stated in the introduction, the production of fungicides is energetically costly and results in the release of a lot of CO_2. Besides, the application of fungicides is costly in terms of money and there is a high risk that pathogens will become resistant. Therefore, farmers and governments strictly regulate the application of fungicides. Fungicides are always applied to plants in a mixture with other fungicides that have different modes of action in the pathogen, thereby making it more difficult for the pathogen to become resistant against fungicides (Gisi & Sierotzki, 2008).

In different countries, systems are used to advise farmers on the correct spraying of fungicides, thereby preventing excessive spraying (HPA, 2014). When the weather is relatively cool (10-15 degrees) and humidity is high, lots of *P. infestans* zoospores can be produced and lots will survive. During such critical weather conditions, chances are thus elevated that a field with potatoes becomes diseased with late blight. When in a certain area a lot of potato plants are present and when already a lot of these plants are infected, the chance that a nearby-located field with potatoes also becomes infected is even higher. Systems measure and forecast these and many more things and then determine if and at what time intervals a farmer should start spraying fungicides to prevent late blight from occurring. It is also determined what type of fungicides can be used best. When the potato plants on a field are growing fast and when pathogen pressure is high, fungicide sprays have to be repeated after several days. The use of these so-called decision support systems, the availability of more environmentally friendly fungicides and research in the population biology of *P. infestans* have resulted in a strong decline of negative environmental effects due to late blight control in agriculture in the Netherlands: the negative environmental impacts of fungicides were reduced by 75% in the period 2003-2013 (WUR, 2013).

7.2 The use of natural compounds

All problems associated with fungicides have triggered people to find other ways to control late blight disease. Besides, organic farms are not willing to use fungicides, so they there is also a need to find other ways to combat late blight.

In lab experiments, it has been shown that many microorganism and compounds derived from them, can enhance the resistance of potato plants against *P. infestans* (Axel *et al.*, 2012). Microorganism in the plant rhizosphere and on the plant shoot can antagonistically interact with *P. infestans*. Microorganism can, for example, compete with *P. infestans* for space and nutrients or they can secrete compounds that harm *P. infestans*. In field experiments, application of microbes to potato plants nevertheless often failed to protect the plants from getting late blight. A reason for this might be that the microbes do not grow all over the plant and are therefore unable to protect all parts of the plant (Axel *et al.*, 2012).

The application of microbe-derived compounds has also been studied in field experiments. Some of these natural compounds enhance the fitness of the plant by a phenomenon called induced systemic resistance (ISR) (Olivieri *et al.*, 2009; Axel *et al.*, 2012). These compounds trigger the innate immune system of the plant to become active, thereby already preparing the plant for an attack by a pathogen. The polyamine spermine, a compound present in all eukaryotic and prokaryotic cells, did enhance the resistance of potato plants against late blight, while several antioxidants, like oxalic acid, did the same, but to a lesser degree: they triggered ISR (Haggag & Abd El-Khair, 2007). Besides, application of the compounds resulted in a lower number of sporangia formed at the leaf surface.

The compounds did change the concentrations of different oxidative enzymes and phenols that are probably involved in the immune response and spermine did significantly stimulate plant growth.

β-aminobutyric acid (BABA), a compound found inside cells, was also able to trigger ISR: plants treated with it had higher concentrations of phenols and phytoalexins in their tubers and a higher activity of proteases, when they were infected with *P. infestans* (Olivieri *et al.*, 2009). BABA diminished the changes of a successful colonization of *P. infestans*.

Despite the resistance-enhancing effects of these natural compounds, they are not applied on farms at a large scale. Several other studies showed that compounds that trigger ISR do negatively affect plant growth and tuber yield (Luna *et al.*, 2014). When these compounds are applied to a plant at wrong times or when they are applied in too high concentrations, overstressing of the plant occurs. Plants do then invest much energy in their immune system, so they have not much energy left to invest in growth, resulting in lower growth rates and tuber yield. Biochemical studies have revealed the pathways by which BABA triggers ISR and represses growth in *Arabidopsis thaliana* (Luna *et al.*, 2014). When a certain protein in this pathway is mutated, BABA-triggered ISR still occurs, while growth is no longer repressed. This opens new opportunities for the application of BABA in late blight control.

7.3 Ground coverage

Another management strategy that is used on farms deals with ground coverage surrounding the potato plants. Ground coverage makes it harder or impossible for *P. infestans* spores to penetrate the soil and reach the potato tubers. When a black polyethylene (plastic) film surrounded the stems of the potato plants in a field, leaving 8 cm directly surrounding the stem uncovered, significantly fewer tubers in the field were affected with late blight, compared to fields were this film was not present (Glass *et al.*, 2001). Besides, when a sort of agricultural textile that was treated with copper hydroxide to allow water to penetrate into the soil surrounded the exact same area of the stem as the black polyethylene film, also significantly fewer tubers were infected. These results indicate that spores do not solely enter the soil near the stem (were more cracks are present in the ground). It is unknown how these two soil coverages do exactly diminish the chances of a successful infection: application of the black film did change soil temperature and dryness, while the complete opposite is true for the agricultural textile. No matter what the reason is, black polyethylene films are used in agriculture to diminish the chance of late blight infections. Nevertheless, the use of these films alone is not enough to achieve low enough levels of late blight occurrence.

It is further good to cover potato tuber that are visible or lie just beneath the ground surface (<15 cm). Tubers that are located at these places have a significant higher chance to be infected with *P. infestans* than tubers at deeper depths (Glass *et al.*, 2001). Planting the potato plants on a big hill, such that all potato tubers are located very deep in the soil, is not necessary, because this does not affect the chance that tubers will become infected with late blight.

7.4 The removal of wild alternative hosts

Many *P. infestans* lineages do not only have potato plants as hosts, but also other *Solanaceae* species (Nowicki *et al.,* 2012). Tomato plants (*Solanum lycopersicum*) are also susceptible to late blight and it thus a good thing to plant potatoes and tomatoes on fields that are not located nearby each other. Besides, several wild *Solanaceae* species function as hosts for most *P. infestans* strains. Black nightshade (*S. nigrum*), bittersweet (*S. dulcamara*) and sticky nightshade (*S. sisymbriifolium*) are all wild hosts for *P. infestans* (Fig. 13) (Flier *et al.*, 2003). *P. infestans* isolates of each host species were able to infect the other wild species and potato plants, causing late blight symptoms to occur. These alternative hosts can thus contribute to the spread of *P. infestans* among potato plants. When inoculating leaves from all these hosts with both the A1 and A2 mating type, oosporogenesis occurred on all leaves. Few oospores (<100/cm^2) were produced on the leaves of the black

nightshade and in the Netherlands, few late blight infections have been reported in black nightshade and bittersweet, suggesting that these plants do not contribute much to late blight disease pressure in potatoes. On the leaves of the sticky nightshade lots of oospores (>300/cm^2) were produced and wild sticky nightshades with late blight symptoms have been reported in the Netherlands, suggesting that this plant has the potential to contribute to disease pressure in potatoes (Flier *et al.*, 2003).

Fig. 13. *Solanum dulcamara*, a wild alternative host for *P. infestans*. *Source: http://www.uniprot.org/*

It is thus recommended to remove these wild alternative hosts when they grow near a farm. Interestingly, the sticky nightshade has certain characteristics that act against potato cyst nematodes, another pathogen that affects potatoes: planting sticky nightshades between potato plants prevents that disease from occurring. This is why some farms plant the sticky nightshade between the potato plants, although this promotes late blight. In that situation, it is recommended to remove the sticky nightshade before the first of September, because that should diminish oosporogenesis in the autumn (HPA, 2014).

7.5 Breeding for resistance

There are, as stated, problems with the abovementioned management strategies and that is why plant breeding to get late blight resistant plants is performed. Most potato cultivars are not resistant to late blight, but there are wild potato species that are resistant to it. These wild species possess R genes that cause the wild species to be resistant. In breeding programs, it is tried to get these R genes in cultivated species, resulting in an improved or completely new cultivar depending on the outcome. This is not easy and can be done in several ways.

7.5.1 Traditional breeding

Traditionally, cultivars are improved by introgression (Dekkers & Hospital, 2002). It is tried to introduce a target gene encoding a trait of interest from an otherwise low-quality line in a high-quality cultivar. Two phases can be distinguished in the process of introgression (Fig. 14). First, a potato cultivar is crossed with a wild species that possesses a trait of interest, for example late blight resistance. It is sometimes possible to hybridize different potato species, though it is complicated since cultivated potatoes are tetraploid, while wild species are often diploid. Therefore, complex breeding schemes are made in which a wild species is first crossed with other wild species to produce intermediate potato hybrids that have the trait of interest and that are also able to cross with the cultivated potato. It is also possible to combine several traits from different wild species in one potato cultivar by using these schemes (for example combining two resistance genes from different wild species). When the cultivar and the wild species are crossed, not only the target gene is introduced in the cultivar, but genes linked to it are also introduced. This is called linkage drag and it often encodes unwanted traits. For example, the form and taste of potato tubers often changes. This is why in the second phase, several generations of backcrosses between the newly generated late blight resistant plants and the cultivated plants are performed. These backcrosses restore large parts of the genome of the cultivar. It is tried to breed plants that are homozygous for the trait of interest, because such plants do only produce offspring with the desired trait: these plants are called pure-bred (Dekkers & Hospital, 2002). It takes at least approximately ten years before unwanted traits are removed and the cultivar is improved enough for registration in breeding registers and commercial use, but on average it can take decades (Haverkort *et al.*, 2009). Since *P. infestans* can rapidly break resistances, much research has focused on finding ways that speed up the process of introducing R genes in potato plants and finding ways to make potatoes durably resistant against late blight.

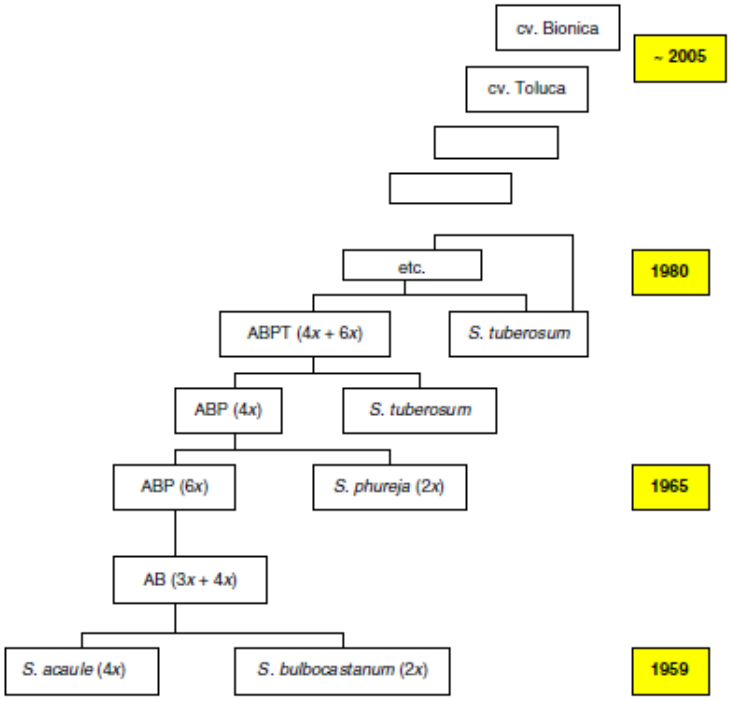

Fig. 14. Breeding scheme used to breed two R genes from *S. bulbocastanum* in *S. tuberosum*. It took 46 years to breed the varieties Bionica and Toluca that have some resistance against late blight. First, *S. bulbocastanum* is crossed with other wild species to produce hybrid intermediates. The final hybrid intermediate (ABP) is crossed with *S. tuberosum*. The product of this cross (ABPT) is crossed again with *S. tuberosum* and the product of that cross is also crossed with *S. tuberosum*, etc. (Haverkort *et al.*, 2009).

7.5.2 Genetic modification

In the Netherlands, Researchers at Wageningen University and Research Centre perform a project called Durable Resistance against *Phytophthora* (DuRPh) in which it is tried to introduce multiple R genes in one potato plant by using cisgenesis and marker-free selection (Haverkort *et al.*, 2009).

The first step in the project is to identify R genes in wild potato species. For that purpose, *P. infestans* is added to a wild potato species to see if the wild species is resistant or not. If the species is resistant, it is necessary to find the genetic architecture underlying the resistance (Vleeshouwers *et al.*, 2008). Often not all individuals in a species are resistant, so crossing of a resistant and susceptible individual results in only a fraction of the offspring being resistant. This fraction can be used to deduce if the resistance is caused by one or more R genes. There are several ways to find the location of possible R genes in the potato genome (Dekkers & Hospital, 2002). A QTL analysis can be performed, as will be explained later. Bioinformatics is nowadays often used to find R genes. R genes have specific DNA-sequences in common, so screening the genome for these sequences results in the finding of many potential R genes. Besides, the genomes of different species can be compared to look for homologies: R genes in one species are sometimes also present in other species in a slightly different form. This approach is sometimes called comparative genomics. After finding the possible R genes, it is checked if the genes are really R genes and if so, what their characteristics are. It is needed to know if an R gene confers resistance to only one strain of *P. infestans* or to several for proper use of the gene in breeding programs. Besides, it is needed to know against what kind of pathogenic effector molecule(s) the R gene acts. To find that out, several things have to be done (Song *et al.*, 2003; Vleeshouwers *et al.*, 2008). First, the genome of *P. infestans* is screened for the presence of effector genes in the exact way as is done with R genes in the potato genome. Actively transcribed effector genes are used in further experiments to see if an R gene acts against them.

Candidate R genes are subsequently cloned in Bacterial Artificial Chromosomes (BACs). A PCR reaction can be performed in which one candidate gene is multiplied from the potato genome and by using restriction enzymes the gene can be subsequently cloned in a BAC. The same is done for the effector genes from *P. infestans*. *Escherichia coli* bacteria are used for storage of the BACs (Haverkort *et al.*, 2009).

The cloned genes can be transferred from *E. coli* to *Agrobacterium tumefaciens*: the genes are then cloned in a special plasmid (the Ti-plasmid) that is present in that species. *A. tumefaciens* can infect plants, which results in the transfer of the cloned genes into the plants cells, creating a genetically modified plant (GM-plant). In this way candidate R genes can be cloned in plants. There are also others methods to transform plants, but they will not be discussed here. If *P. infestans* isolates are added to the transformed plants and a hypersensitive reaction occurs, that confirms that the gene is indeed an R gene. One can add different strains of *P. infestans* to the plant to see to what strains the gene confirms resistance. Moreover, one can transform a plant with an R gene and an effector gene, so that they are both present at the same time in the plant genome. When a hypersensitivity response occurs, it can be concluded that the R gene acts against the effector that has also been placed in the plant (Fig. 15). By trying different kinds of combinations, one can find out the exact spectrum of effectors that an R gene works against, an approach sometimes called effector genomics (Vleeshouwers *et al.*, 2008). In this way a broad-spectrum R gene from the wild potato species *Solanum bulbocastanum* was cloned that conferred resistance to all tested *P. infestans* strains, including a strain that was resistant against a lot of other R genes (see below) (Song *et al.*, 2003).

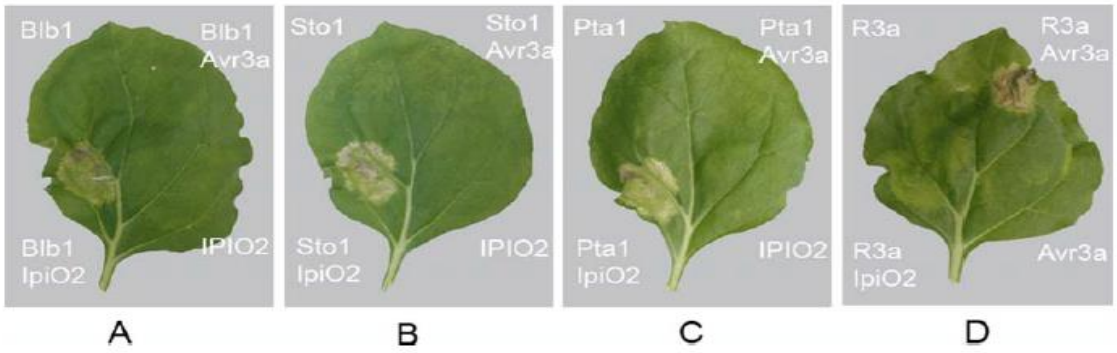

Fig. 15. The basic idea of finding R genes. Leaf cells of the tobacco plant (*Nicotiana benthamiana*) were transformed with the RXLR-effector ipiO2 and different R genes. In the upper left part of each leaf cells are located that were transformed with an R gene, but that were not transformed with the effector ipiO2. Logically, no HR occurred. In the upper right part, cells transformed with an R gene and the Avr3a effector are present. R3a recognized Avr3a: HR did occur (this was already known and was done as a control). In the down right part, cells transformed with only the effector ipiO2 are present (or Avr3a for leaf **D**). Logically no HR occurred. In the down left part cells transformed with an R gene and ipiO2 are present (or Avr3a for leaf **D**). HR occurred in leaves **A**, **B** and **C**. Therefore, it can be concluded that the R genes named Blb1, Sto1 and Pta1 all can recognize ipiO2 (Vleeshouwers *et al.*, 2008).

The second step in the project is to transform a potato cultivar with more than one R gene (at least three), a process called gene pyramiding (Haverkort *et al.*, 2009). It is best to introduce more than one R gene, because that diminishes the chances for *P. infestans* to break the R genes and it makes potato plants therefore durably resistant. In the past, eleven R genes from the wild potato species *Solanum demissum* have been cloned all independently in different potato cultivars and all these resistances have been overcome by some *P. infestans* strains. There is even one strain that has been able to overcome all eleven R genes. Several R genes from wild species are cloned in one BAC and this is called a gene cassette. To transform a potato plant with the genes, a part of the stem or leaf is cut from the plant and put on an agar dish. Then the plant cells on the dish are transformed using *A. tumefaciens*. Since plant cells are totipotent, the cells can grow into new potato plants.

On average, only 3% of the plant cells is transformed. To select these cells, PCR reactions are performed to check for the presence of the genes (Haverkort *et al.*, 2009). No selection markers, such as antibiotic markers, are thus used to select the transformed cells. Therefore the project is called marker free. The selected cells are grown into plants by adding hormones and these plants are subjected to lots of tests. In laboratory and field experiments, plants are infected with *P. infestans* and with other pathogens to see if the plants are resistant and plant growth, tuber yield and diverse

physiological parameters are measured. Besides, the genomes of the plants are screened for the presence of wanted or unwanted genes, using the MAS technique that will be described later. Besides, a test panel checks the taste of the potato. Only plants that succeed through these tests are selected to produce a new generation of plants and these are also subjected to tests. This can go on for several generations.

To make it even harder for *P. infestans* to break the resistances, it is tried to design different gene cassettes of R genes and generate transformed plants with different R genes in their genomes (Haverkort *et al.*, 2009). If different "variants" are used in different places and times, resistances in potatoes will probably be more durable. Besides, it is useful to make many gene cassettes, because new cassettes can then be used when the older ones do not work anymore.

The DuRPh-project has achieved many results. Several R genes have been cloned from wild species and several gene cassettes have been made and successfully cloned into plants: these plants were still "true to type" (Haverkort *et al.*, 2009). Nevertheless, it turned out to be hard to design cassettes containing four or more R genes: these constructs were not stable and too big to be transported into plants with *A. tumefaciens* (Haverkort, personal communication).

Very recently, a gene was found in the wild potato species *Solanum microdontum* that encoded a membrane receptor that recognized several elicitins (Du *et al.,* 2015). Elicitins are extracellular effectors secreted by *P. infestans* that have functions in amongst others lipid binding. They are very conserved among *Phytophthora* species and evolve slowly, just like Pep-13 discussed earlier. The receptor, which is not an R protein (R proteins recognize fast-evolving intracellular effectors) was able to recognize multiple elicitins from different *Phytophthora* species and induced the HR. When transferred in a potato cultivar, resistance against late blight was significantly enhanced. Since elicitins evolve slowly, the receptor gene might be used in combinations with R genes to design durably resistant potato plants.

An important objective of the project is to promote the discussion about genetic modification in agriculture. Broadly, there are two types of genetic modification: cisgenesis and transgenesis (Fig. 16) (Schouten *et al.*, 2006). When genes from related lineages or species are manually added to the genome of a plant, this is called cisgenesis. For these genes, it is also possible to get in the plant by crossbreeding in a natural way: it does not make sense if this is easy or hard. The transformation of potato cultivars with genes from related wild potatoes, such as in the DuRPh project, is an example of cisgenesis. Since the project is marker free, no selection marker genes that are derived from non-related organisms are used. The transformation of a plant with genes from non-related organisms is called transgenesis. For these genes, it is impossible to get them in the plant in any natural way.

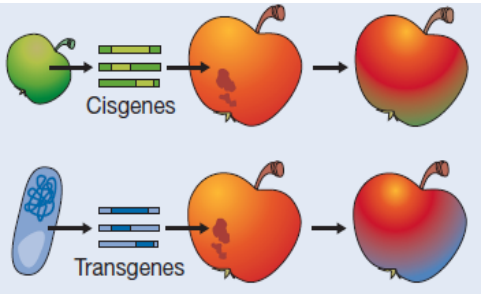

Fig. 16. Cisgenesis uses genes from relatives, while transgenesis uses genes from non-relatives (Schouten *et al.*, 2006).

A famous example of transgenesis is the Fortuna potato (Gillund *et al.,* 2013). This potato cultivar was made by adding two R genes from the wild potato species *S. bulbocastanum* to the plant. This project was not marker free. A gene, derived from the non-related plant species *A. thaliana*, which encodes a specific herbicide resistance was transformed along with the two R genes in the potato plant as a selection marker. When a specific herbicide is sprayed on the agar dish with transformed and not-transformed plant cells, only the transformed plant cells will survive and be selected. Nevertheless, this results in the presence of a gene from an unrelated species in the potato and therefore the process is called transgenesis.

In Europe, the release of cisgenic and transgenic plants on the market is strictly regulated. Many tests should be performed by many different institutes and these tests should all indicate that the plants have no negative effects on the environment, biodiversity and human health (Schouten *et*

al., 2006). It can take years before such a cultivar is finally allowed entry to the European market. Moreover, GM-crops meet strong opposition in Europe. Organizations like Greenpeace performed all kinds of actions to prevent the application of the Fortuna potato on the European market and they had success: the company behind Fortuna withdrew from the European market (Gillund *et al.,* 2013).

Especially in the Netherlands, diverse research institutes have lobbied for less strict rules for the release of cisgenic plants on the market (Schouten *et al.*, 2006). It is said that cisgenic plants are fundamentally different from transgenic plants: cisgenesis does not change the gene pool of a species, while transgenesis does. Besides, in traditional breeding unwanted genes are also bred in the cultivar. The presence of these genes might have negative effects on the environment or human health. In cisgenesis, no unwanted genes are placed in the cultivar. Therefore cisgenesis might even be safer than traditional breeding. Moreover, there are GM-techniques, like protoplast fusion and mutagenesis that are not regulated by extremely stringent rules. Finally, it is said that cisgenesis is the only fast enough way to make plants that have a high productivity and low susceptibility to disease to meet the worlds growing demand for food.

The only DNA fragments that are not derived from related species and that are transferred to a plant during cisgenesis are some parts of the Ti-plasmid of *A. tumefaciens*. This is used as one of the arguments by opponents of cisgenesis (Schouten *et al.*, 2006). Moreover, many opponents say that cisgenesis violates the integrity of life: people should not manipulate DNA (Lammerts van Bueren *et al.*, 2008). Some people go far in expressing their opinion: fields with cisgenic plants that were used for the DuRPh-project have been destroyed several times.

Several Dutch research institutes have concluded that assessment of all new plant cultivars should be based on the specific characteristics of that variety (the product) and not on the process that was used to make the new cultivar (Prins & Kok, 2010). It might be best to stop treating GM-plants differently than traditionally bred plants: all should be assessed using the same tests and all should be regulated using the same rules. The European Food Safety Authority (EFSA) is of the opinion that regulations regarding cisgenic plants can indeed be relaxed. On the basis of the findings of all these institutes, the Dutch government actively lobbies for the relaxation of the European rules regarding cisgenic plants: the government sees no arguments why the European Commission should not relax the rules (Mansveld, 2013). It is expected that the European Commission will decide whether or not to change the rules in 2015.

7.5.3 Marker-assisted selection: breeding with some help

Organic agriculture opposes cisgenesis on ethical grounds: manipulating DNA violates the integrity of life (Lammerts van Bueren *et al.*, 2008). Nevertheless, organic agriculture has major problems to control late blight disease and many organic farms go bankrupt due to the devastating effects of late blight. In the Netherlands, the area on which organic potatoes are grown declined from 1.555 ha in 2002 to 1.217 ha in 2007 (Lammerts van Bueren *et al.*, 2008). The demand for organic potatoes is nevertheless increasing more than ever in the Netherlands and therefore it is necessary to improve and speed up the traditional breeding procedure.

In the Netherlands, Researchers at Wageningen University and Research Centre perform a project as part of a green breeding program called Bioimpuls (Lammerts van Bueren *et al.*, 2008). The project uses molecular markers to perform QTL analyses and MAS selection, which speed up the traditional breeding process. The overall aim of the project is to identify new R genes in wild species and try to breed multiple R genes in an already existing potato cultivar, thereby improving the cultivar without using genetic engineering. After one R gene has been successfully bred in a cultivar, another R gene is bred in the cultivar and so on. To achieve this, new molecular markers are generated.

For the identification of R genes a quantitative trait locus (QTL) analysis can be performed to find the chromosomal location of these genes, as stated earlier. For a QTL analysis many markers are used. Markers are DNA sequences with a known location in the genome. Examples of markers are

DNA-satellites and SNPs. These DNA stretches always have more than one allele and it can be visualized in some way what kind of allele this is (i.e. a or A). DNA-satellites can be visualized by, amongst others, RFLP or AFLP techniques and SNPs can be visualized by sequencing the marker (see Cooke & Lees, 2004 for detailed information about the different kinds of markers and how to visualize them). Genes that are located nearby a marker are linked to that marker: they tend to be inherited together with the marker. Genes that are located far away from each other are often not inherited together due to much recombination between homologues chromosomes. This phenomenon is called linkage disequilibrium and it is used in a QTL analysis.

In a QTL analysis, first an individual with resistance to late blight is crossed with an individual that is susceptible to late blight (Fig. 17A). This cross produces the F1 generation: all these individuals possess one entire chromosome from the resistant parent plant and one from the susceptible parent plant. F1 individuals are then crossed with other F1 individuals to produce the F2 generation. During this cross the chromosome from the resistant parent and the chromosome from the susceptible parent recombine. Some of the F2 individuals will be resistant against late blight, while others will be susceptible. It is scored what allele every resistant individual has for every marker and this is subsequently also done for the susceptible individuals. If it turns out using statistics that resistant individuals often have a different allele for a certain marker than susceptible individuals have, it can be said that a gene that is involved in resistance is located near that marker. Since it is known what the location of the marker is, the region were the R gene is located is probably nearby that region. To find the exact location of the R gene, one could do another QTL analysis with markers that are all located in that region or one could sequence the region and see what genes are located there. Subsequently, one could mutate the genes to check if they are really R genes or one could clone them as described earlier.

In the Bioimpuls project, a technique called marker-assisted selection (MAS) is used (Fig.17B). In this technique markers are used to deduce if an individual has a certain trait or not. From a QTL analysis one learns which markers tell you something about the trait. For example, a certain marker may have the A-allele in resistant plants and the a-allele in susceptible plants. If it is then unknown if a plant is resistant or susceptible to late blight and one finds the A-allele it can be concluded that the plant is most probably resistant. MAS is thus an indirect way to find out what traits an individual has.

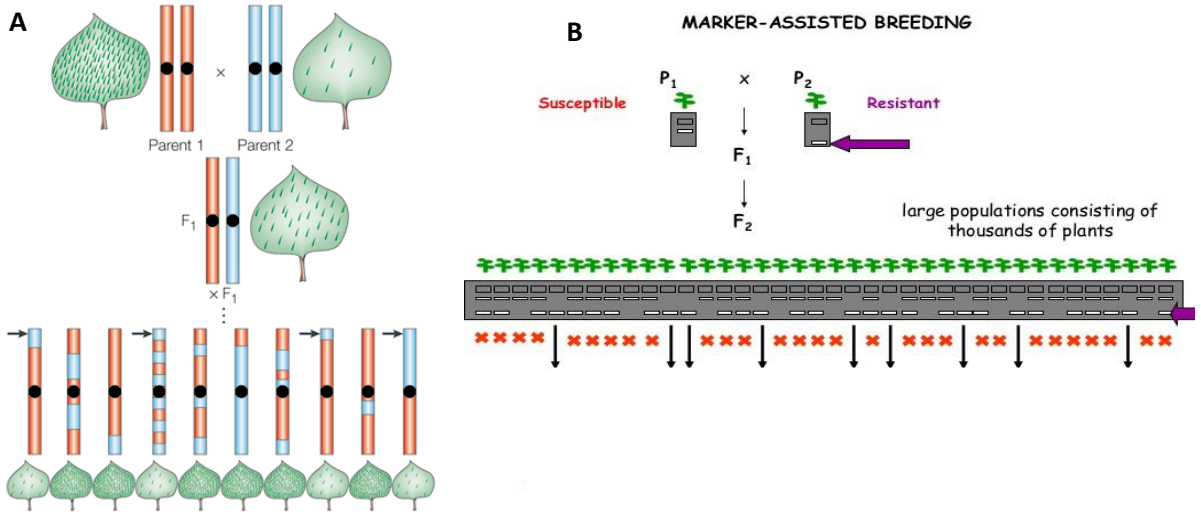

Fig. 17. A: The principle of the QTL analysis, as explained in the text. If you want to know, for example, where the genes that determine the number of spikes on leaves are located in the genome, you should perform this cross. In the F2 generation, recombination between the chromosomes from parent 1 and parent 2 can be observed. The arrow denotes the part of the chromosome were a gene that determines the number of spikes is probably located. Individuals with a low number of spikes always have the blue marker allele, while individuals with many spikes always have the red marker allele (Mauricion, 2001). **B**: The principle of MAS, as explained in the text. Plants that are resistant to a certain disease happen to have a different isozyme pattern than plants that are susceptible to the disease. When crossing a susceptible cultivar and a wild resistant species, one could select resistant plants in the F2 progeny by looking at the isozyme pattern of the plants. The purple arrow indicates the plants that have an isozyme pattern that is characteristic for resistant plants. These plants should be used for further breeding. *Source: Presentation DNA labs, Lammerts van Bueren, 2013.*

In recent years high throughput techniques for screening markers are being developed, which has made MAS a commonly used technique in plant breeding.

Besides genomic markers, other markers can be used in MAS. For example, one might use physiological or phenotypic markers (Cooke & Lees, 2004). If it turns out that there is a correlation between a physiological or phenotypic parameter and resistance, measuring the parameters can reveal whether a plant is resistant or not. To give an example, it might be that resistant plants always have a certain enzyme present in high amounts. If high amounts of the enzyme are detected in a plant, it can be concluded that the plant is resistant.

MAS speeds up traditional breeding by making the breeding procedure more efficient (Dekkers & Hospital, 2002). First, MAS helps in the design of proper breeding schemes. As stated earlier, breeding genes from wild potatoes in potato cultivars is difficult and often requires intermediate hybrids. If one knows exactly what kind of wanted and unwanted genes these species possess, one might design breeding schemes in such a way that there is a high chance for wanted traits and a low chance for unwanted traits to be bred in the cultivar. Second, when performing backcrosses MAS is used for screening individuals on the traits they possess. It is possible to "read" the genome of an individual and conclude from that what kinds of different traits and alleles the individual has. In that way it is possible to quickly determine whether the individual is appropriate to be used for further crossings or not. For example, one might screen individuals and select individuals that are homozygous for the trait of interest, since these individuals are pure-bred. Therefore, it is no longer needed to perform a lot of different kinds of time-consuming and costly tests. Field experiments with agricultural crops can namely only be performed in the growing season and that does limit speeding up the breeding process.

Nevertheless, some physiological and phenotypic experiments are still always performed in breeding programs (Dekkers & Hospital, 2002). The phenotype of an individual is the result of interactions between genotype and environment and it is thus possible that individuals with the same genotype have a different phenotype due to differences in the environment. Moreover, genes do interact with each other and this might also influence the phenotype. Besides, there are some statistical and technical problems associated with QTL analyses and MAS.

The Bioimpuls project has achieved some results. Potato varieties with multiple resistance genes have been bred. Plants with four resistance genes have been bred successfully (Lammerts van Bueren, personal communication).

7.6 An integrative approach: the best way to control late blight

In 1998, the Dutch agricultural sector faced serious problems with controlling late blight. Besides, the sector failed to become more environmentally-friendly. Therefore, the sector developed an integrative approach to control late blight called the Masterplan *Phytophthora* (HPA, 2014). Several research programs, such as the DuRPh- and Bioimpuls-project are set up as part of this plan. The Masterplan gives farmers advice on the proper control of late blight. It recommends the use of as many management strategies as possible to prevent late blight from occurring. Besides the use of fungicides, natural compounds, ground coverage, removal of alternative hosts and resistance breeding, as already discussed, the Masterplan also mentions other management strategies to prevent late blight from occurring. Some examples of these are the following.

- Harvesting potatoes should be done carefully to prevent seed tubers from wounding. Spores of *P. infestans* can easily penetrate potato tubers through wounds. Besides, farmers should take care to remove all potato tubers from the field.
- Diseased potato tubers should be thrown away and when a lot of potatoes on a field are diseased, it is best to throw all potatoes away.
- Potatoes tubers can be diseased without seeing any disease symptoms. Therefore, potato tubers should be stored in a cold and dry place to prevent zoospores from spreading from an infected tuber to other still healthy tubers.

- It is very important to cover refuse piles or to remove them since it has been shown that these piles are important sources of late blight infections (Zwankhuizen *et al.*, 1998). Farmers risk fines if do not cover or remove the refuse piles.
- When plants are found diseased with late blight, the foliage should be removed to prevent further spread of the disease. Moreover, to prevent the migration of the disease from foliage to tubers, it is a good thing to remove the foliage 7-14 days before harvest (Tsedaley, 2014).
- When approximately 7% of the potatoes plants on a field are infected with late blight, farmers have to remove all plants. If this is not done, farmers risk fines.
- If a field has been cropped with potatoes in one year, it is best to wait three to four years at minimum before cropping potatoes again in that field. This diminishes the chances of late blight infections caused by oospores.

Some other preventive actions that farmers in the Netherlands often perform, but that are not explicitly mentioned in the Masterplan are the following ones.

- Field with potato should have good water infiltration and drainage characteristics (Tsedaley, 2014). These fields are relatively dry, which makes it harder for zoospores to survive and reach new potato plants.
- Fields should not be excessively irrigated, since the presence of water can enhance zoospore survival and motility (Tsedaley, 2014). Besides, it is not recommended to irrigate the fields during the night and early morning, because *P. infestans* prefers to sporulate in the dark.
- Potatoes should be planted in rows that parallel the prevailing winds (Tsedaley, 2014). In this way, foliage dries quickly and the spread of spores by means of wind is made more difficult.

It is very important for farmers to follow up the advices of the Masterplan *Phytophthora* because only the combination of different management strategies can result in the effective control of late blight.

8. Conclusions and recommendations

This bachelor thesis dealt with late blight disease in potato plants (*Solanum tuberosum*). Plants quickly die when they have late blight and the disease can rapidly spread between plants, causing the disease to have devastating effects in agriculture. Late blight is caused by *Phytophthora infestans*, a hemibiotrophic oomycete, that can reproduce asexually and sexually. In recent decades, management strategies for controlling late blight are increasing not effective anymore, resulting in increasing late blight related problems. The pathogen is becoming resistant against the currently used management strategies. The most effective management strategy for late blight control is an integrative one: different actions that disrupt the life cycle of *P. infestans* in different ways should be combined to prevent late blight from occurring and to diminish the chances that the pathogen becomes resistant against them. Currently, research focuses on finding ways to improve existing management strategies and tries to design new management strategies.

The integrative approach used in the Netherlands seems successful in terms of clarity. It is clear for farmers where they can find information about late blight and what they should do to prevent late blight from occurring. Besides, farmers are warned when late blight problems are expected, so they can take precautions and it is checked if farmers follow up advices and rules. Research is always a part of an integrative approach, resulting in more knowledge about the life cycle of *P. infestans* and the breeding of plants that are expected to be durably resistant against late blight. Integrative approaches used in many other countries also seem to be successful.

Efforts should be made to maintain the coordinated way in which countries like the Netherlands approach late blight. Nevertheless, there are several recommendations to make for further research and for the improvement of late blight management strategies.

8.1 Recommendations for further research

It is important that research focuses on elucidating the fundamental aspects of the life cycle of *P. infestans*. If it is known exactly which molecules and genes play which roles in which life stadia, new windows might be opened for the design of new fungicides or for the search for biological compounds that specifically target one or more of such molecules or genes. In the past, this has already been successfully done. It should be tried to develop fungicides that have no detrimental side effects and that can be used for a long time: it is thus preferred to design fungicides that have more than one target in the pathogen. Far too less is known about oosporogenesis in *P. infestans*, although oospores cause major problems in agriculture. Research should focus much more on the sexual life cycle of *P. infestans*.

Moreover, research should focus on studying the effector molecules that are secreted by *P. infestans* during an infection. If it is known in molecular and genetic detail how effectors work, this might generate new opportunities to combat late blight. This needs some explanation.

Suppose that an effector molecule binds to a receptor on the surface of a plant cells and that this binding triggers an intracellular signaling pathway, which finally leads to a changed cellular physiology. In the absence of the pathogen, the signaling pathway is also used by the plant, but in another way. Let us assume that the pathway then ensures that cellular physiology stays the same. The effector thus interferes with an already existing signaling pathway in the plant cell: the pathogen parasitizes it for its own good. The physiological change brought about by triggering the pathway helps the pathogen to infect the plant and that is thus not a good thing from the plants perspective. To prevent the cellular change from happening, two things can be done by the plant in theory. The plant can use R proteins that guard the molecules of the signaling pathway: the R protein can recognize the effector and start an immune response. The signaling pathway thus stays the same, but it is protected by R genes. Another thing a plant can theoretically do is change the signaling pathway in such a manner that the effector molecule is not able to trigger it anymore. This might be achieved by mutations in the genes that are involved in the pathway. In this scenario, the plant changes the signaling pathway, but does not use R genes.

Most research focuses on finding R genes in wild potato species and the subsequent breeding of these R genes in a plant. It is recommended that research starts exploring the possibilities to make or breed resistant plants by changing or interfering with signaling pathways. If resistant wild potato species are found with a different signaling pathway than susceptible plants, it might be useful to find out what does exactly differ between the pathways. If, for example, one gene is mutated in the resistant plant, it can be tried to breed that gene into a potato cultivar, thereby hopefully making the cultivar resistant. Nevertheless, there might be some serious problems associated with changing signaling pathways, explaining why not much research has focused on it. Since a molecule can function in different signaling pathways, a change of the molecule can affect all these pathways. To keep all pathways functioning properly, other molecules may also have to change. Therefore many genes might have to be mutated to not change the functioning of the cell and these all have to be bred in the plant cultivar to not change its phenotype. It might therefore be more feasible to breed with R genes and other genes that function in the immune response. Research should maintain its main focus on finding new R genes and breeding them in plants, but it should also make some efforts in exploring changes in pathways.

Recent research has started to isolate viruses from *P. infestans* (Cai *et al.*, 2013). It has yet to be elucidated what characteristics these viruses have. If the viruses are able to kill *P. infestans*, new windows for controlling late blight might be opened.

Furthermore, research has to continue elucidating how certain microorganisms or biological compounds can trigger induced systemic resistance (ISR) and how that affects plant growth. As said, it is now known by what pathways β-aminobutyric acid (BABA) triggers ISR and negatively affects plant growth. This will generate opportunities for diminishing the negative effects on plant growth of such compounds.

Also, the interactions between wild potato plants and *P. infestans* should be investigated more. This may lead to new insights for the control of late blight. Besides, it is thought that not all wild potato species have yet been discovered. Therefore, the search for new wild potato species should continue. The International Potato Centre, which collects, stores and grows wild potato species, should continue its activities. Moreover, conservation biologists have to investigate the possibilities for the protection of wild potato species. It might be worth investigating whether hotspots with many potato species, such as those in Peru, can be declared nature reserves.

Finally and very importantly, the problem of late blight should be investigated in a more interdisciplinary way: researchers in areas like plant pathology, molecular plant biology, plant physiology, plant breeding and botany have to work together to find good solutions for late blight.

8.2 Recommendations to improve late blight management strategies

A recommendation for the Dutch government is that they should always strictly control potato farms. It is a good thing if all farms are controlled on a yearly basis. The controls should be performed during the winter months, to see if the potatoes are stored in the right way and if refuse piles have been removed or covered.

The spread of different genotypes and the presence of oospores should be carefully monitored every year. Nowadays, the presence of oospores is not routinely checked. Farmers should be sent newsletters every year with updates about changes in the population of *P. infestans* and advices on how to deal with these changes.

Late blight control strategies differ between countries. There is a strong need for internationalizing the control of late blight: *P. infestans* does not care about country borders. The European Union has designed some policies regarding the use of fungicides, GMOs and food safety, but not specifically about late blight control strategies. In Europe, there is an organization called EuroBlight in which researchers from different countries discuss late blight control strategies. It is a good thing if that organization gets more tasks. It should design a policy or an advice that can be used in all European countries, thereby standardizing the control of late blight. The organization should also monitor the different types of *P. infestans* across Europa and their spread. This information should be available for free for farmers in all countries.

It might even be better to establish a global organization that addresses late blight problems: both developed and developing countries have major problems to control late blight. Besides, monitoring the spread of late blight at a global scale may help in the better control of the disease. At the moment there are several international organizations that deal with agricultural problems at a global scale, but none of them does explicitly deal with late blight. Most of these organizations deal with food security and hunger problems in developing countries. For that reason, it might be that developed countries do not want these organizations to design international late blight policies or advices. In the past several international conferences with researchers from all over the world have taken place, in which control strategies were discussed. These conferences have not been taken place in the last few years. It is thus strongly recommended to organize new conferences in which researchers and policymakers from different countries are present. During such conferences, discussions should be held about internationalizing the control of late blight. If countries want to internationalize the control of the disease, working groups should be established that need to find out how this can be best achieved.

Will we ever be able to combat *Phytophthora infestans*? Will we ever be able to stop the economic and environmental impacts and human losses of late blight disease and increase food security? Will we ever be able to feed a future world with nine billion people? Science is contributing greatly to a better understanding of *Phytophthora infestans* and has already contributed to the control of the devastating pathogen. With the enormous progress that science is making through in the last decades, I hope that we can answer yes to all three questions.

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