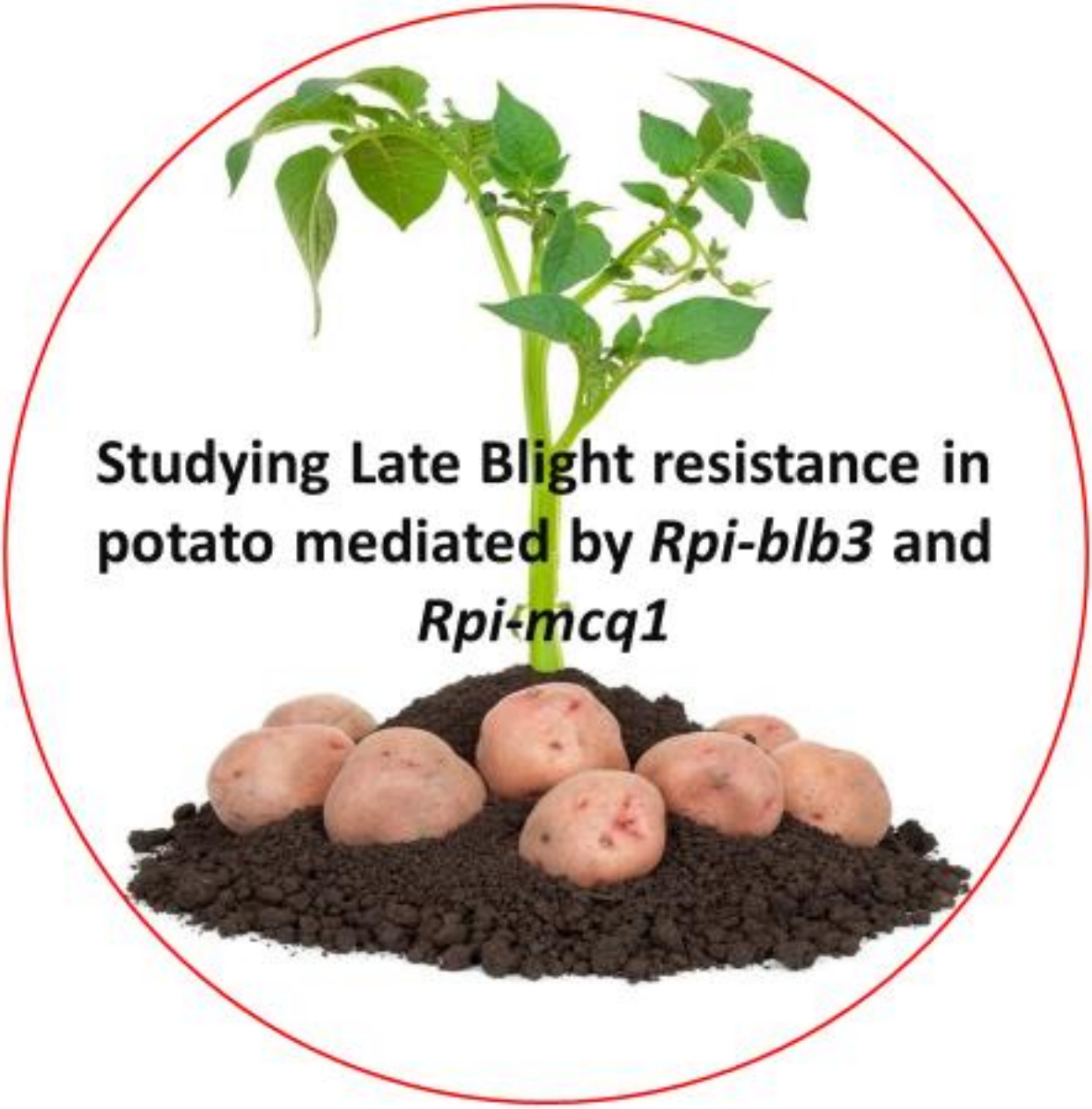


Studying Late Blight resistance in potato mediated by *Rpi-blb3* and *Rpi-mcq1*

Md Mazadul Islam

Msc. Minor Thesis
Plant Sciences
Plant breeding and genetic resources
Wageningen university

Supervisors:
Dr. Vivianne Vleeshouwers
Carolina Aguilera Galvez

Examiner:
Dr. Evert Jacobsen

WAGENINGEN
UNIVERSITY & RESEARCH

Copyright ©

Niets uit dit verslag mag worden verveelvoudigd en/of openbaar gemaakt door middel van druk, fotokopie, microfilm of welke andere wijze ook, zonder voorafgaande schriftelijke toestemming van de hoogleraar van de Laboratory of Plant Breeding van Wageningen Universiteit.

No part of this publication may be reproduced or published in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without prior written permission of the head of the Laboratory of Plant Breeding of Wageningen University, The Netherlands.

MSc minor thesis (PBR 80424)

Studying Late blight resistance in potato mediated by *Rpi-blb3* and *Rpi-mcq1*

By

Md Mazadul Islam

Registration number: 880606383020

Plant Breeding Department

Wageningen University and Research

January 2018

Supervisors

Dr. Vivianne Vleeshouwers

Carolina Aguilera Galvez

Examiner

Dr. Evert Jacobsen

Abstract

Potato late blight is the most important worldwide threat for potato production, caused by a notorious oomycete *Phytophthora infestans*. Globally, a huge amount of fungicides is applied in the potato field to control this disease, but difficult to control. The pathogen is too much fast evolving and single gene resistance breeding has failed to provide a durable resistant potato crop. So, control of this disease is a big challenge for the resistance breeder. To obtain a durably resistant variety, stacking of multiple broad-spectrum *R* genes is advised. Two *R* genes, *R2* (also referred as its homolog *Rpi-blb3*) and *Rpi-mcq1,* were isolated from wild *Solanum* species of Mexico and Peru, respectively and characterized their resistance spectrum. These two *R* genes show a different resistance profile to a range of *P. infestans* isolates where *R2* provides a slightly broader resistance spectrum and isolate NL07434 being a representative of the isolates that are virulent on *Rpi-mcq1* and avirulent on *R2*. The *R2* and *Rpi-mcq1* recognized the *P. infestans* effector AVR2 but the effector AVR2-like is recognized by *Rpi-mcq1* only. The first research question was to unravel the differences in disease test result with the isolates, stable transformants with *R2* and *Rpi-mcq1* were tested with three effectors (PITG_10640, PITG_04099 and PITG_22935) that were upregulated during the infection of NL07434 and for recognition by R2. The same *R* genes and effectors were also co-agroinfiltrated in *Nicotiana benthamiana*, *Solanum tuberosum* cv. Désirée and cv. Bintje to validate the result. We found that both *R* genes conferred cell death responses upon agroinfiltration with PITG_10640, PITG_04099, but not with RD2 (PITG_22935). The same set of effectors also caused cell death when co-expressed with *R3a*, which indicates that the cell death is not specific of *R2* and *Rpi-mcq1*. A second topic was to determine the virulence profile of Peruvian *P. infestans* isolates on Désirée-*R2*, Désirée-*Rpi-mcq1* and Désirée-*Rpi-hcb* plants. Because *Rpi-mcq1* and its homolog *Rpi-hcb* were identified in wild *Solanum* from Peru and the virulence profile of those *R* genes was always tested with isolates of the Netherland, Europe and Mexico. We found that *R2* and *Rpi-mcq1* showed the same resistances profile against the isolates from Peru.

Keywords: AVR2, effector, isolates, Potato, *Phytophthora infestans*, *R* gene, and transformant.

Contents

Abstract iv

1. Introduction 1

1.1. Potato 1

1.2. Potato late blight 1

1.3. Mechanisms of plant-pathogen interaction 2

1.4. Breeding for *P. infestans* resistance 4

1.5. Effectors of *Phytophthora infestans* 6

1.6. Background of the research 7

1.7. Aim of the project 8

2. Materials and methods 9

2.1. ATTA (*Agrobacterium tumefaciens*-mediated transient transformation) 9

2.1.1 Plant materials 9

2.1.2 *Agrobacterium tumefaciens* cultures 9

2.1.3 Agroinfiltration (ATTA) 10

2.1.4 Experimental setup 11

2.2. Virulence profile of P. infestans isolates 12

2.2.1 Plant materials 12

2.2.2 *P. infestans* isolates 12

2.2.3 Detached Leaf Assay (DLA) 13

3. Results 14

3.1. Co-agroinfiltration response of effectors and *R* genes in *N. benthamiana*, potato cultivar Bintje and Désirée 14

3.2 Single infiltration response of Désirée-R2, Désirée-Rpi-mcq1 and wild species 15

3.3. Virulence profile of *P. infestans* isolates 16

4. Discussion 20

4.1 Co-agroinfiltration response in *N. benthamiana*, Bintje and Désirée 20

4.2 Single infiltration response in Désirée-R2, Désirée-Rpi-mcq1 and wild species 20

4.3 *Phytophthora infestans* isolates and disease test 21

5. Conclusion and Recommendations 23

6. Acknowledgement 24

7. References 25

Appendices 30

1. Introduction

In this chapter potato and potato late blight disease is introduced and followed by an overview of the mechanisms of plant-pathogen interaction. Later sections are introduced with breeding for late blight resistance, background and aim of the research.

1.1. Potato

Potato (*Solanum tuberosum* L.) is an exigent food crop in the world food production (Kim et al., 2011), domesticated in South America and expanded to extensive parts of Europe and Asia. In 2014, world total potato production was 325 million tonnes and its total coverage was 19 million hectares of land (FAOSTAT, 2017). Currently, it grades the third position after rice and wheat regarding human consumption (Haverkort et al., 2009). The utilization of potato is not only confined to food crops but also has a contribution in non-food production, especially the production of alcohol, starch, glue and many other industrial products (Li et al., 2009, Du 2014).

Like other crops, different types of biotic and abiotic factors hamper the potato production. Drought, salinity, temperature, light etc. are the abiotic factors. On the other hand, the biotic factors are pests and pathogens which can reduce the crop yield dramatically. Among the biotic stresses, late blight is one of the most devastating diseases of potato, which results in 16% global yield losses and € 5.2 billion financial losses (Haverkort et al., 2009).

1.2. Potato late blight

The late blight of potato is caused by a notorious plant pathogen, *Phytophthora infestans.* It is a hemi-biotrophic oomycete. The pathogen is well known for causing the Irish potato famine in the 1840s, but still, now it is a threat to global food security. One hypothesis narrated that the centre of origin of *P. infestans* is Mexico (Goodwin et al., 1994; Grünwald and Flier, 2005), but a competing hypothesis debates that the Andes of South America is the centre of origin of both potato and *P. infestans* (Gómez-Alpizar et al., 2007). In favourable environmental conditions, the late blight disease can cover the whole field within a few weeks (Vossen et al. 2005). The disease not only invade the leaves, stems and tubers of potato but also the other species of Solanaceae species (Birch et al. 2012). The pathogen is so virulent and aggressive, and it has the potentiality to adapt to the resistant plant (McDonald & Linde, 2002; Fry, 2008). For this reason, it is considered as a model organism for studying pathogens of oomycete.

From the genomic point of view, a deep analysis of *P. infestans* genome illuminate the reasons for their virulence and aggressiveness. The genome of *P. infestans* was sequenced in last decade. It has a genome of 240 Mb which is most complicated and largest genome compared to the other three related species of Phytophthora. The genome showed a

strange structure that helps the pathogen to adapt to a wide range of hosts (Haas et al., 2009; Raffaele et al., 2010). The genome consists of high gene density regions and low repeat regions (gene islands), as opposed to regions with low gene density (gene deserts) and repeat-rich regions. The repeat-rich and sparse area of the gene contains the virulence factors and effector genes that helps them to evade the resistant gene.

P. infestans can reproduce sexually which increases the diversity of the pathogen and worldwide survivability (Fry, 2008). But the asexual clones are responsible for late blight epidemics which spread and amplified by the sporangia of infected plants over aerial dispersion (Fry et al., 2009). Infected plants and tubers can act as a source for the spread of spores in the growing season and in another season.

1.3. Mechanisms of plant-pathogen interaction

Plants are sessile organisms, they totally depend on their immune system to encounter the pathogen attack. The plant immunity is inherent, possess receptor protein from plasma membrane and cytoplasm that recognizes the pathogen effector protein and bind them to initiate the immunity. Effectors are the proteins, secreted by the pathogen to break the plant defence system and initiate the disease (Niks et al., 2011). The active immune system of plants briefly consists of two layers (figure 1) (Jones and Dangl 2006; Win et al., 2012). The first layer consists of transmembrane pattern recognition receptors (PRRs) on the surface of the cell. PRRs can detect apoplastic microbial effectors called microbe or pathogen-associated molecular patterns (MAMPs or PAMPs). This type of defence is known as PAMP-triggered immunity (PTI). The PAMPs are an essential and conserved molecule for the pathogen, For instance, chitin in fungus and flagellin in bacteria. The PRRs mediated defence is a commonly broad spectrum and conserved, provides resistance or basal immunity to non-adapted pathogens.

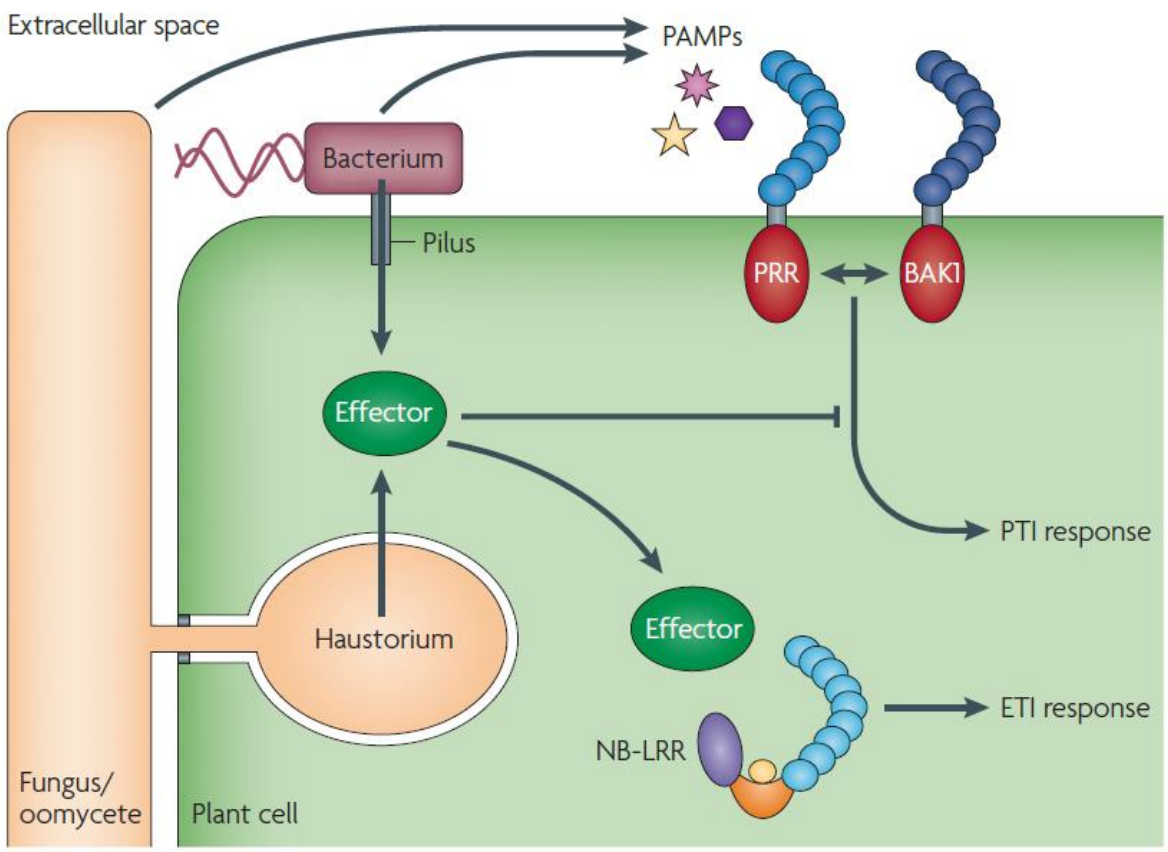


Figure 1. Principles of plant immunity system. Apoplastic effectors and PAMPs are recognized by PRR protein while NB-LRR can recognize the intracellular or cytoplasmic effectors (Dodds & Rathjen, 2010)

The second layer of immunity works when the first layer of defence fails. In this defence system, the pathogens cytoplasmic effectors (e.g. RxLR effectors for *Phytophthora*) are recognized by the resistance (R) proteins. The R proteins are coiled-coil nucleotide binding leucine-rich repeats (CC-NB-LRRs) domain (Jones and Dangl, 2006). This type of immunity known as effector-triggered immunity (ETI), is fast evolving and has a narrow spectrum (Jones & Dangl, 2006; Macho & Zipfel, 2014). But the pathogens are very clever and they diversify their effectors to suppress the plant's immunity or recognition, results in effector-triggered susceptibility (ETS). This interplay of resistant and susceptible plants is well described in the Zigzag model of Jones and Dangl (2006), where more layers of defence are described (figure 2).

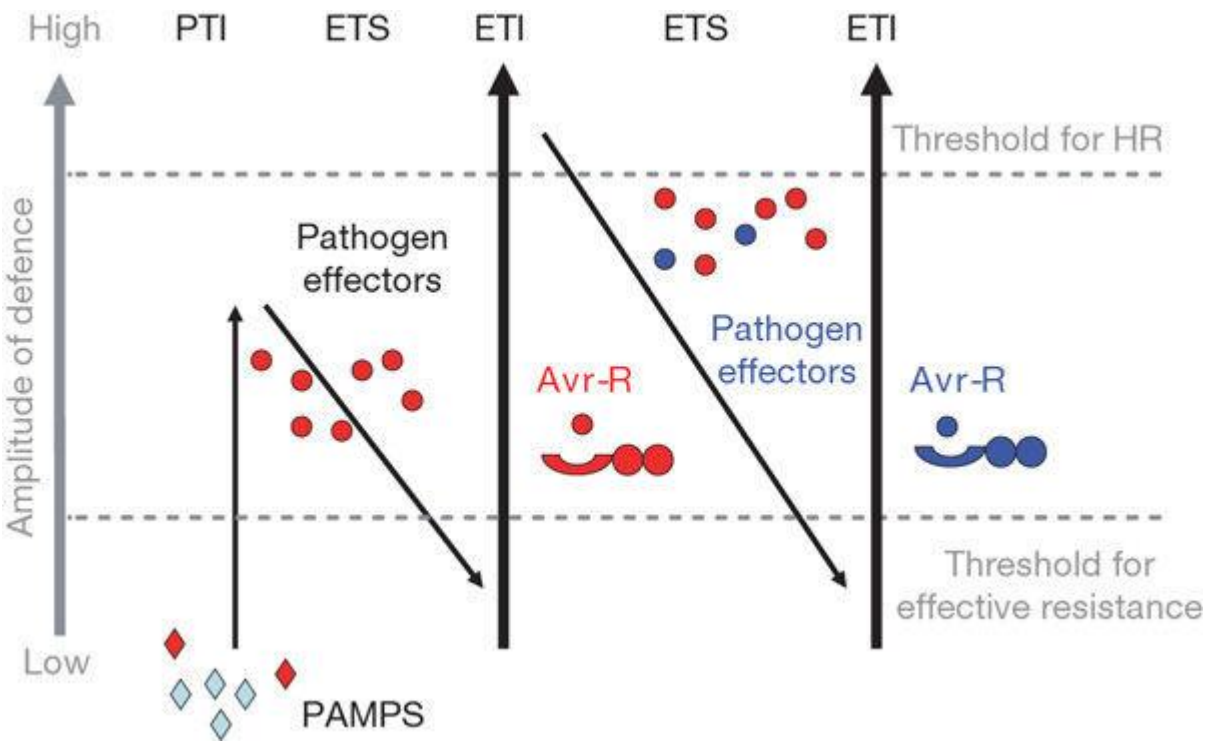


Figure 2. Zigzag model of plant immunity system illustrating the multiple phases of immunity. PTI, ETI generates immunity to plant and ETS makes susceptible to the pathogen. The AVR-R pair controls the amplitude of defence (Jones & Dangl, 2006).

Pathogen effectors recognition by the NB-LRR protein provides a strong defence against the pathogen, characterized by a programmed cell death, called the hypersensitive response (HR) (Vleeshouwers et al., 2011). When effector molecules are recognized by the R(NB-LRR) proteins, they are known as avirulence protein (AVR), causing a-virulence. Gene-for-gene model describes the interaction between R and Avr-proteins (Flor, 1971), where a specific R protein recognizes the corresponding Avr-protein and results in Hypersensitive resistance (HR). It indicates the diversity of *R* genes and the high specificity of HR.

1.4. Breeding for *P. infestans* resistance

In the field of potato breeding, the major focus is to develop potato varieties resistant to *P. infestans*. Introduction of *R* genes from wild species into a commercial variety provides resistance against the pathogen, a promising and sustainable method of resistance breeding. But resistance shows two types, *i.e.* vertical resistance and horizontal resistance. When resistance is controlled by single *R* gene then it is called specific or vertical resistance and it has provided less durable resistance (Malcolmson and Black, 1966). On the other hand, horizontal resistance is controlled by polygenes or is a quantitative trait locus which called (QTL) provides a durable resistance to multiple races of a pathogen (Gebhardt, 1994; Tan et al., 2008).

The wild Mexican species *S. demissum* is a good source of *R* genes and multiple *R* genes (*R1-R11*) were identified from this species, which showed a broad-spectrum resistance against *P. infestans*. However, *P. infestans* is fast evolving, can generate diversified new strains to break resistance (Foster et al., 2009). That's why it is very difficult to control *P. infestans* by single *R* gene. As a result, the breeding program has been designed for a durable resistant scheme against the late blight. Consequently, a stacking of multiple broad-spectrum *R* genes is considered to provide a durable resistance (Zhu et al., 2012; Jo et al., 2014).

Several *Solanum R* genes that confer resistance to *Phytophthora infestans* were identified from *S. bulbocastanum, S. edinense, S. schenckii, S. hjertingii, S. stoloniferum and S. venturii* (Vleeshouwers et al., 2011) (figure 3). Recently *Rpi-mcq1* has been cloned from *S. mochiquense* (Jones et al., 2014) and *Rpi-amr3i* gene from *S. americanum* (Witek et al., 2016). Another *R* gene, *Rpi-hcb,* is a homolog of *Rpi-mcq1* has been cloned from *S. huancabambense* (Aguilera-Galvez et al., unpublished). *Rpi-ber* and *Rpi-chc1* has been cloned from *Solanum berthaultii (*Rauscher et al., 2006) and *S chacoense,* respectively. Among all the *R* gene families, the *R2* family is extremely diverse and situated in chromosome IV on a locus for major late blight (MLB) resistance. Members of this family differently recognize the effector AVR2 (Vleeshouwers et al., 2011). *Rpi-blb3* recognizes the AVR2, but the virulent allele of AVR2 is AVR2 like (A2L) is not recognized by *Rpi-blb3*. So, *Rpi-blb3* carrying plants infected by *P. infestans* isolates expressing AVR2 like (A2L) (Saunders et al., 2012). The genes *R2* and *Rpi-mcq1* were identified from wild potato species of Mexico and Peru, respectively. They showed a differential disease resistance spectrum against different isolates of *Phytophthora infestans*. *Rpi-mcq1* recognizes both AVR2 and AVR2 like and is located in chromosome IX. So, it indicates that both genes are from different gene families. However, the recognition of *R* gene is related to the corresponding effectors of *Phytophthora infestans.*

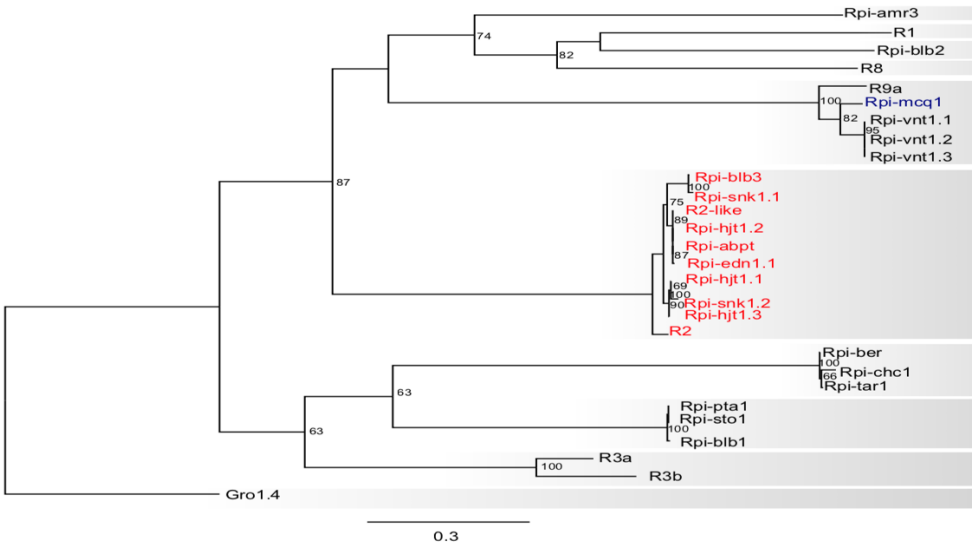
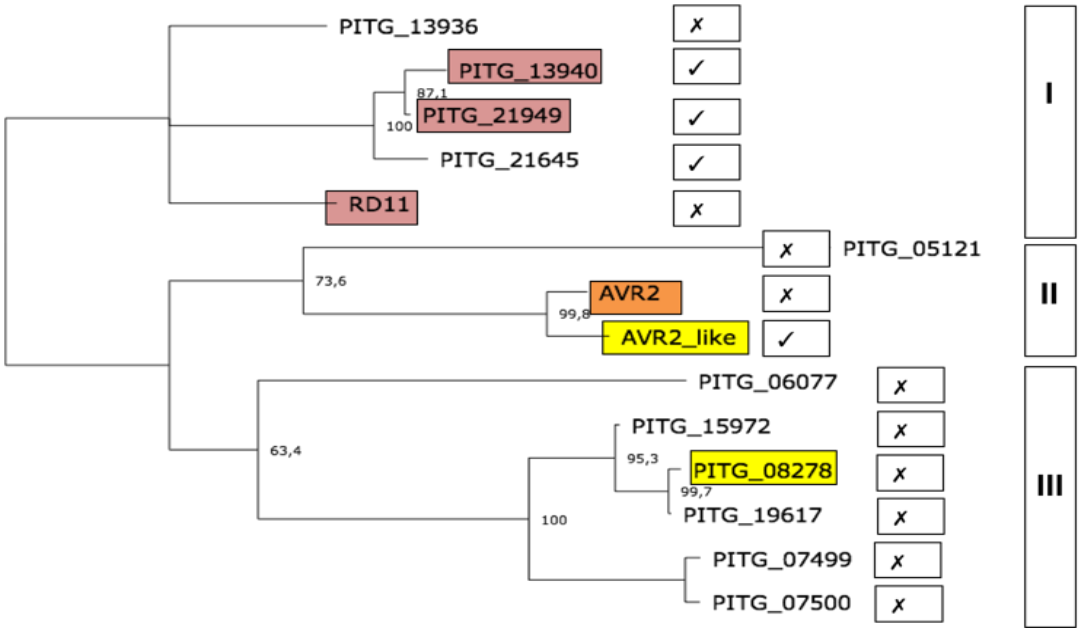


Figure 3. Phylogenetic tree of *Solanum R* genes showing *R2, Rpi-mcq1* and its homologs (Aguilera-Galvez et al., submitted)

1.5. Effectors of *Phytophthora infestans*

Both apoplastic (outside of the cell) and cytoplasmic (inside the cell) effectors are secreted by *Phytophthora infestans* (Kamoun, 2006; Hein et al., 2009; Whisson et al., 2016). Cytoplasmic effectors include RXLR peptide motif with C-terminal effector domain. RXLR motif is necessary for the secretion of the protein into the host plant cell (Whisson et al., 2007, 2016; Dou et al., 2008; Anderson et al., 2015). The AVR gene works inside the host cell, exists in the repeat-rich region and is considered to upregulate in the biotrophic stage of infection. Recent studies have found that many RXLR effectors from *P. infestans* accumulate in the haustoria and secret the effector from this structure after penetration (Whisson et al., 2016, Wang et al.,2017). The mode of effector secretion is still unknown, but the delivery of effector inside the host cell was clearly observed (Whisson et al., 2016). However, RXLR effectors can be used to detect the specific recognition of functional *R* genes in the potato plant through the effectoromics approach.



X means not expressed and √ means expressed during potato infection with *P. infestans* isolate Blue13. That means PITG 13940, PITG 21645 and Avr2 like are the only 3 AVR2 effectors expressed.

Figure 4. Phylogenetic relationship and clustering of AVR2 effector family. The expression of the effectors after potato infection with the isolate blue13 are indicated (Aguilera- Galvez et al., in preparation)

The AVR2 effector family of *P. infestans*, is an exceptionally differing group of 14 variants, grouped into three clades (figure 4). Various members of this family are upregulated during the infection of potato by the isolate blue13. Among them, the AVR2, AVR2-like and RD11 are differentially recognized by the *R2* and *Rpi-mcq1* (Aguilera Galvez et al., submitted). The AVR2 is recognized by both, but AVR2-like is recognized by only *Rpi-mcq1* and RD11 recognition is vice versa with the result of AVR2-like. Additionally, the results from another experiment showed, some interesting RXLR effectors of *P. infestans* were upregulated

during potato infection, which is not a member of the AVR2 family (Aguilera-Galvez et al., in preparation).

1.6. Background of the research

Carolina Aguilera Galvez is a PhD student studying late blight resistance with the *R* genes, *R2* (*Rpi-blb3*) and *Rpi-mcq1*, which recognize AVR2. The *R* genes were identified in the wild *Solanum* species through effectoromics approach. This technique is a high throughput functional genomic tool in this era (Vleeshouwers et al.,2011). Two *R* genes (*R2* and *Rpi-mcq1)* were identified in wild *Solanum* from Mexico and Peru respectively and were transferred to the susceptible commercial cultivar, Désirée. The *Rpi-hcb* is a homolog of *Rpi-mcq1* and was also transferred to Désirée. However, those *R* genes display a differential disease resistance with three isolates tested: NL07434, NL09096 and NL12003 (Table 1).

Table 1. Differential disease profile of *P. infestans* isolates on *R2* and *Rpi-mcq1* plants

P. infestans isolate	Désirée- *R2*	Désirée - *Rpi-mcq1*
NL07434	R	S
NL12003	R	S
NL09096	R	S

On the other hand, *R2* and *Rpi-mcq1* recognize members of the AVR2 family, and the specificity of recognition is distinct (table 2). It indicates that the difference in disease test is not related to presence or absence of AVR2 or AVR2-like. RD11 could explain the different resistance phenotypes and this is also following in another experiment.

Table 2. Specificity of recognition of some AVR2 members by *R2* and *Rpi-mcq1*

Effectors	*R2*	*Rpi-mcq1*
AVR2	Recognized	Recognized
AVR2-like	Not recognized	Recognized
RD11	Recognized	Not recognized

To find out more about this, the isolate NL07434 was checked during potato infection with two reference strain (figure 5). The result showed that some interesting effectors were upregulated during the infection which was not in the AVR2 family. The effectors were PITG_22935, PITG_21422, PITG_04099, PITG_18405 and PITG_06246. The effector PITG_22935 is a member of RD2 effector family, and the effector PITG_18405 has a 99% of identity with PITG_10406. Based on the availability of the effector they were selected for the further test. To validate these results, we would like to test whether those candidate effectors are differentially recognized by *R2* and *Rpi-mcq1*.

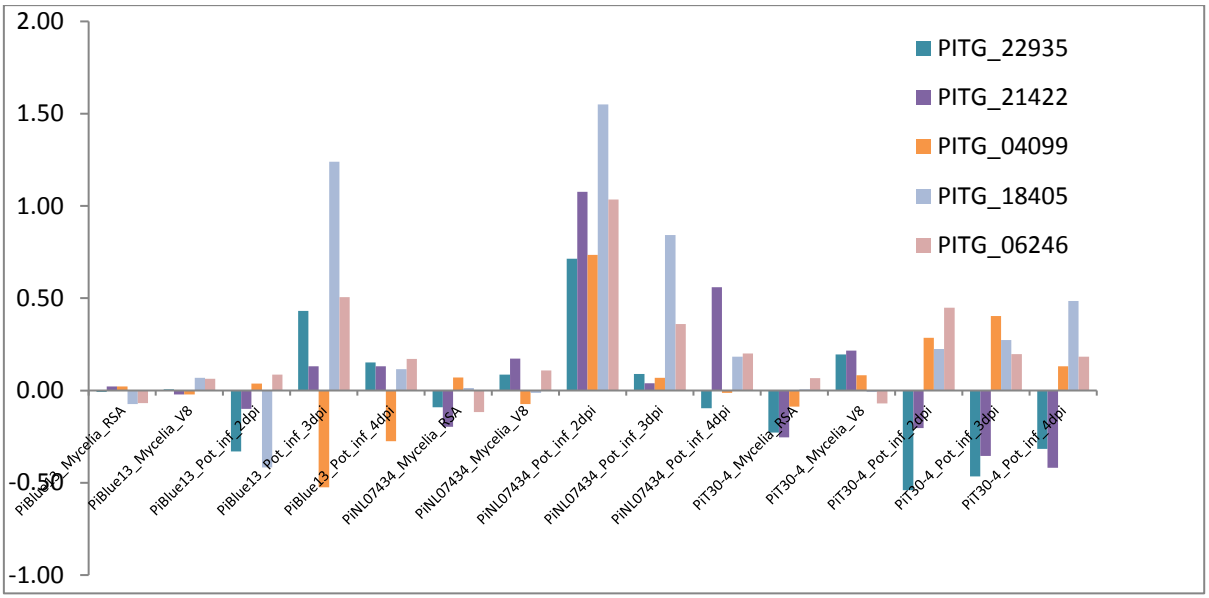


Figure 5. *P. Infestans* effectors upregulated during infection on potato (microarray data)

Additionally, we are also interested in the evaluation of *P. infestans* isolates from Peru, in order to determine the virulence of the isolates in potato plants harboring *Rpi* genes from Peru. In previous experiments of this project, virulence profile of 18 *P. infestans* isolates has been determined. Three of them are differentially recognized by *R2* and Rpi-mcq1, but most of them are from the Netherlands, Europe and Mexico (Aguilera-Galvez et al., Submitted). Therefore, it is important to test more diverse isolates to see a broader virulence profile. Based on these research outcomes the aim of this project is selected.

1.7. Aim of the project

The aim of the project is to unravel the differential disease resistance spectrum that is conferred by *R2* and *Rpi-mcq1*, respectively. Another aim is to quantify the virulence profile of *P. infestans* isolates from Peru on Désirée- *R2*, Désirée-*Rpi-mcq1* and Désirée-*Rpi-hcb.*

The following research questions were developed to meet the above-mentioned objectives.

Q1. Can *R2* and *Rpi-mcq1* recognize the PITG_10640, PITG_04099 and RD2 effectors?

Co-agroinfiltrations of candidate effectors and *R* genes in *N. benthamiana,* potato cv. Bintje and Désirée; single infiltrations of the candidate effectors in Désirée-*R2,* Désirée-*Rpi-mcq1*, and wild species (source of the mentioned *R* genes) will be conducted to find out the answer.

Q2. Potato plants containing *R* gene from Peru can confer resistant to *P. infestans* from same geographic location?

P. infestans isolates from Peru will be tested on Désirée-*R2*, Désirée-*Rpi-mcq1* and Désirée-*Rpi-hcb* plants by detached leaf assay.

2. Materials and methods

This chapter describes the materials and the methods used to achieve the research objectives and to accomplish this thesis. The first section explains the *Agrobacterium tumefaciens*-mediated transient transformation followed by virulence profile of *P. infestans* isolates. It describes the plant materials, pathogen isolates, experimental setup, data collection, methods and statistical analysis.

2.1. ATTA (*Agrobacterium tumefaciens*-mediated transient transformation)

2.1.1 Plant materials

The plant materials used in this experiment can be found in table 1. All the plant materials except *Nicotiana benthamiana* were clonally propagated *in vitro* from the stock material by cutting. The clones were grown in MS20 medium (Murashige and Skoog, 1962) for two weeks in a climate chamber. The media was prepared with 4.4 g of Murashige and Skoog basal salt mixture accompanied with all vitamins, 20 g of sucrose and 8 g of micro agar per liter and the pH was adjusted to 5.8. Moreover, 2 ml per litre plant preservative mixture (PPM^{Tm}) was used in the media to prevent the microbial infection in the culture. After two weeks of growth in a climate chamber, the plants were transferred into the greenhouse and grown in small trays for two weeks. After that, the plants were transferred into the big pot of 2kg of soil and grown for two weeks in a restricted area of Unifarm greenhouse. The *N. benthamiana* plants were provided by the Unifarm employees and 45 days old potato plants were used for ATTA.

Table1. List of plants used in the experiment of ATTA

Abbreviate/ Common name	**Species**	**Remarks**	**source of *R* gene**
Désirée-*R2*	*Solanum tuberosum*	Gene inserted	*Solanum bulbocastanum*
Désirée-*Rpi-mcq1*	*Solanum tuberosum*	Gene inserted	*Solanum mochiquense*
Mcq717-3	*Solanum mochiquense*	Donor of Rpi-mcq1	
Hcb353-8	*Solanum huancabambense*	Donor of *Rpi-hcb*	
Hjt349-3	*Solanum hjertingii*	plant contains *R2*	
Snk213-1	*Solanum schenkii*	plant contains *R2*	
edn150-1	*Solaum edinense*	plant contains *R2*	
Désirée	*Solanum tuberosum*	Control plant	
Bintje	*Solanum tuberosum*	Control plant	
Tobacco	*Nicotiana benthamiana*	Model plant	

2.1.2 *Agrobacterium tumefaciens* cultures

The *R* genes and effectors used in this study were previously cloned in a binary vector and used for *A. tumefaciens* transformation. All the *A. tumefaciens* clones (table 2) were available in the lab and provided by Carolina for this experiment.

Table 2. List of *Agrobacterium tumefaciens* cultures with vector for Agroinfiltration study

R gene/ Effector	Agrobacterium strain	Vector
R2 (*Rpi- blb3*)	AGL1	pBinplus
Rpi-mcq1	AGL1	pBinplus
R3a	AGL1	pBinplus
AVR2	AGL1	pK7WG2
PITG_10640	AGL1	pK7WG2
PITG_04099	AGL1	pK7WG2
RD2 (PITG_22935)	AGL1	pK7WG2
AVR*3a*	AGL1	pK7WG2
Empty vector (-)	AGL1	pK7WG2

2.1.3 Agroinfiltration (ATTA)

According to the protocol of Du and Rietman et al. (2014), the agroinfiltration assays were conducted in this study. There was an exception from the protocol that, the cultures of *R* gene and effectors were started from the glycerol stock into 3ml LB (Lysogeny broth) medium with all necessary antibiotics (streptomycin, kanamycin, chloramphenicol and carbenicillin) according to the vector. All other steps were performed according to the protocol. Hypersensitive response based on the cell death was scored after 4 or 5 days of agroinfiltration. The scoring was done on a scale of 0-2 (figure 6).

Score 0 **0%** | **Score 0.5** **25%** | **Score 1** **50%** | **Score 1.5** **75%** | **Score 2** **100%**

Figure 6. Hypersensitive response (HR) scoring scale for ATTA test

2.1.4 Experimental setup

Co-agroinfiltrations with *R2* and *Rpi-mcq1* in *Nicotiana benthamiana,* potato cv. Bintje and Désirée were performed by following combinations.

Table 3. Combination of *R* and *AVR* gene pairs for Co-agroinfiltrations with optical density

Plant 1 (3 leaves per plant) *R2* + effectors

Number	**Effector**	**Final OD for Bintje and Désirée**	**Final OD for *N. benthamiana***
1	*R2* + AVR2	0.2	0.5
2	*R2* + PITG_10640	0.2	0.5
3	*R2* +PITG_04099	0.2	0.5
4	*R2* +RD2	0.2	0.5
5	Negative control	0.2	0.5
6	R3a-Avr3a	0.2	0.5

Plant 2 (3 leaves per plant) *Rpi-mcq1* + effectors

Number	**Effector**	**Final OD for Bintje and Désirée**	**Final OD for *N. benthamiana***
1	*Rpi-mcq1* + AVR2	0.2	0.5
2	*Rpi-mcq1* + PITG_10640	0.2	0.5
3	*Rpi-mcq1*+PITG_04099	0.2	0.5
4	*Rpi-mcq1* +RD2	0.2	0.5
5	Negative control	0.2	0.5
6	R3a-Avr3a	0.2	0.5

Plant 3 (3 leaves per plant) Control effectors with *R3a*

Number	**Effector**	**Final OD for Bintje and Désirée**	**Final OD for *N. benthamiana***
1	*R3a* + AVR2	0.2	0.5
2	*R3a* + PITG_10640	0.2	0.5
3	*R3a*+PITG_04099	0.2	0.5
4	*R3a* +RD2	0.2	0.5
5	Negative control	0.2	0.5
6	R3a-Avr3a	0.2	0.5

Plant 4 (3 leaves per plant) Control just effectors

Number	**Effector**	**Final OD for Bintje and Désirée**	**Final OD for *N. benthamiana***
1	AVR2	0.2	0.5
2	PITG_10640	0.2	0.5
3	PITG_04099	0.2	0.5
4	RD2	0.2	0.5
5	Avr3a	0.2	0.5
6	R3a-Avr3a	0.2	0.5

Single infiltrations with the candidate effectors were done in Désirée-*R2,* Désirée-*Rpi-mcq1*, and wild species with the following combinations. Because these plants already contain the *R* genes, only AVR genes were necessary for agroinfiltrations.

Table 4. Effectors with optical density for Agroinfiltration in potato

3 plants per genotype (3 leaves per plant)

Number	Effectors	Final OD for the potato plants
1	AVR2	0.2
2	PITG_10640	0.2
3	PITG_04099	0.2
4	RD2	0.2
5	Negative control	0.4
6	R3a-Avr3a	0.4

Three replications were performed for all combinations of *N. benthamiana* and potato plants at three different times of one-week interval.

2.2. Virulence profile of *P. infestans* isolates

2.2.1 Plant materials

In this experiment, the plant materials were also prepared according to the same procedure of the previous experiment of ATTA by cutting in *vitro*.

Table 5. List of plants used in the experiment of detached leaf assay (DLA)

Abbreviate/Common name	Species/source of *R* gene	Remarks
Désirée-*R2*	*Solanum bulbocastanum*	Gene inserted
Désirée-*Rpi-mcq1*	*Solanum mochiquense*	Gene inserted
Mcq717-3	*Solanum mochiquense*	Donor of *Rpi-mcq1*
Hcb353-8	*Solanum huancabambense*	Donor of *Rpi-hcb*
Double transformant- 2	*R2/ Rpi-blb3* and *Rpi-mcq1*	Gene stacking
Double transformant- 3	*R2/ Rpi-blb3* and *Rpi-mcq1*	Gene stacking
Double transformant- 4	*R2/ Rpi-blb3* and *Rpi-mcq1*	Gene stacking
Désirée-*Rpi-hcb1.1* transformat-7	*Solanum huancabambense*	Gene inserted
Désirée-*Rpi-hcb1.1* transformat-8	*Solanum huancabambense*	Gene inserted
Désirée-*Rpi-hcb1.1* transformat-22	*Solanum huancabambense*	Gene inserted
Désirée-*Rpi-hcb1.2* transformat-2	*Solanum huancabambense*	Gene inserted
Désirée-*Rpi-hcb1.2* transformat-3	*Solanum huancabambense*	Gene inserted
Désirée-*Rpi-hcb1.2* transformat-56	*Solanum huancabambense*	Gene inserted
Désirée	*Solanum tuberosum*	Control plant

2.2.2 *P. infestans* isolates

Five *P. infestans* isolates were used in this experiment, provided by Carolina from plant breeding departments isolate stock. Among the isolates, three isolates were new in this research and other two isolates (IPO-0 and PIC99183) were used as a control. The isolates NL07434 and NL09096 were used as an extra test for the genotypes, to confirm the previous experimental result. All *P. Infestans* isolates were propagated 10 days before the experiment in rye media.

Table 6: List of *P. infestans* isolates with their origin and virulence profile

Name of isolate	**Origin**	**Virulence profile**		
		Désirée	***R2***	***Rpi-mcq1.1***
IPO-0	Netherlands	S	R	R
PIC-99183	Netherlands	S	R	R
NL07434	Netherlands	S	R	R
NL09096	Netherlands	S	R	R
Peru 4	Peru		Unknown	
Peru 29	Peru		Unknown	
Peru 42	Peru		Unknown	

R designates resistant and S designates susceptible

2.2.3 Detached Leaf Assay (DLA)

Detached Leaf Assay was done to determine the pathogenicity of *P. infestans* isolates to the potato plant materials of this experiment. Seven weeks old plant, grown in the greenhouse was used for detached leaf assay. The *P. infestans* isolates were grown on the rye agar medium ten days before the DLA. In cold water, the fresh spores were harvested and 10 spores per 0.2 mm were considered as an optimum concentration for inoculation. Isolates inoculation and DLA procedures were followed by the protocol of Vleeshouwers et al. (1999). Three leaflets of one leaf was inoculated with ten droplets of inoculum of each isolate. Three leaves per genotype were inoculated with one isolate in each tray. The whole experiment was repeated three times to keep three biological replications. The lesion sizes were measured from the inoculated leaves three times, usually at four, five and six days after inoculation with electronic callipers, connected to a laptop. The lesion growth rate (LGR) was calculated from the lesion size by a linear linear regression over time (Vleeshouwers et al.,1999). The infection efficiency (IE) was estimated by the percentage growing lesions comparative to total number of inoculation spot per leaf per genotype. Then the infection efficiency (IE) and lesion growth rate (LGR) were analysed by the software Genstat 18th edition.

3. Results

In this chapter, first, the results of co-agroinfiltration and agroinfiltration are presented. The next section represents the results of detached leaf assay.

3.1. Co-agroinfiltration response of effectors and *R* genes in *N. benthamiana*, potato cultivar Bintje and Désirée

To study the recognition spectrum of different effectors by *R2* and *Rpi-mcq1*, the effectors were co-infiltrated with *R2*, *Rpi-mcq1* and *R3a* in *N. benthamiana*, Bintje and Désirée. The cell death responses were scored five days post infiltration. In all replication sets, *N. benthamiana* didn't show any response, except for the positive control. *R2, Rpi-mcq1* and *R3a* caused cell death with the effectors AVR2, PITG_10640 and PITG_04099 in both Bintje and Désirée (figure 7 & 8). The effector RD2 was not recognized by any *R* gene. Unfortunately, the empty vector (negative control) showed some responses in potato plant especially in Désirée, which was not expected. It indicates the sensitivity of Désirée to *Agrobacterium*. The results also showed that the effectors PITG_10640 and PITG_04099 unexpectedly developed some HR response without any *R* gene. It can also happen because of the treatments with empty vector alone. The positive control, *R3a-Avr3a* pair was recognized and developed HR response in both Bintje and Désirée. The HR index score is presented in Appendix 1 and the HR pictures are presented in appendix 3. The experiment was done three times and each replicate consisted of one plant with three leaves.

The Co-agroinfiltration result showed the cell death response for *R2*, *Rpi-mcq1* and *R3a* with AVR2, PITG_10640 and PITG_04099. But the result for control *R3a* fails because of AVR2 recognition.

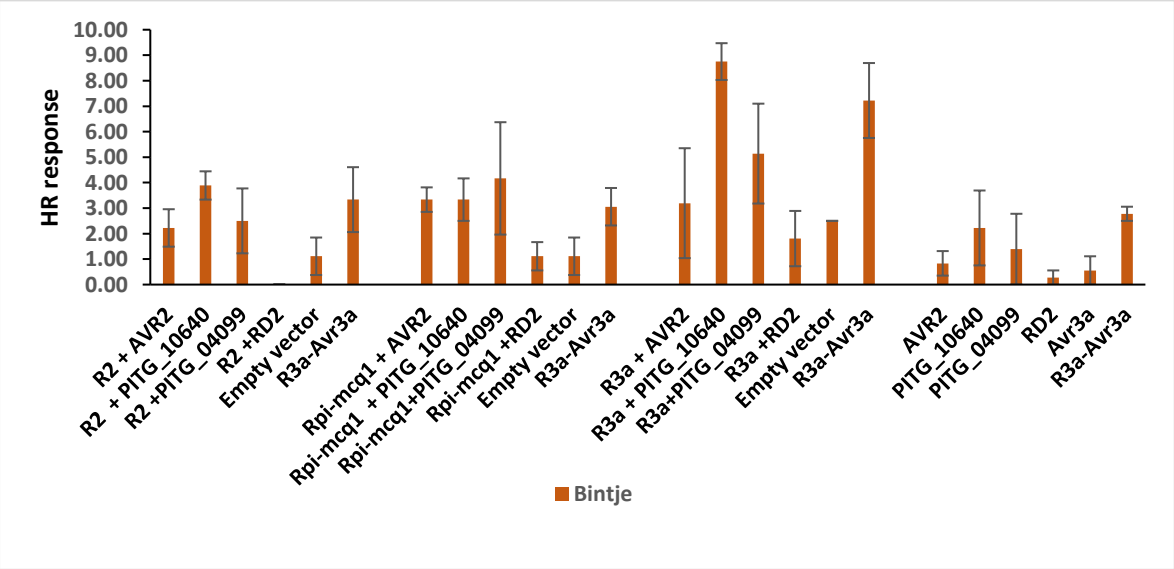


Figure 7. Co-infiltration of *R2*, *Rpi-mcq1* and *R3a* with different effectors in potato cultivar Bintje showing the HR responses

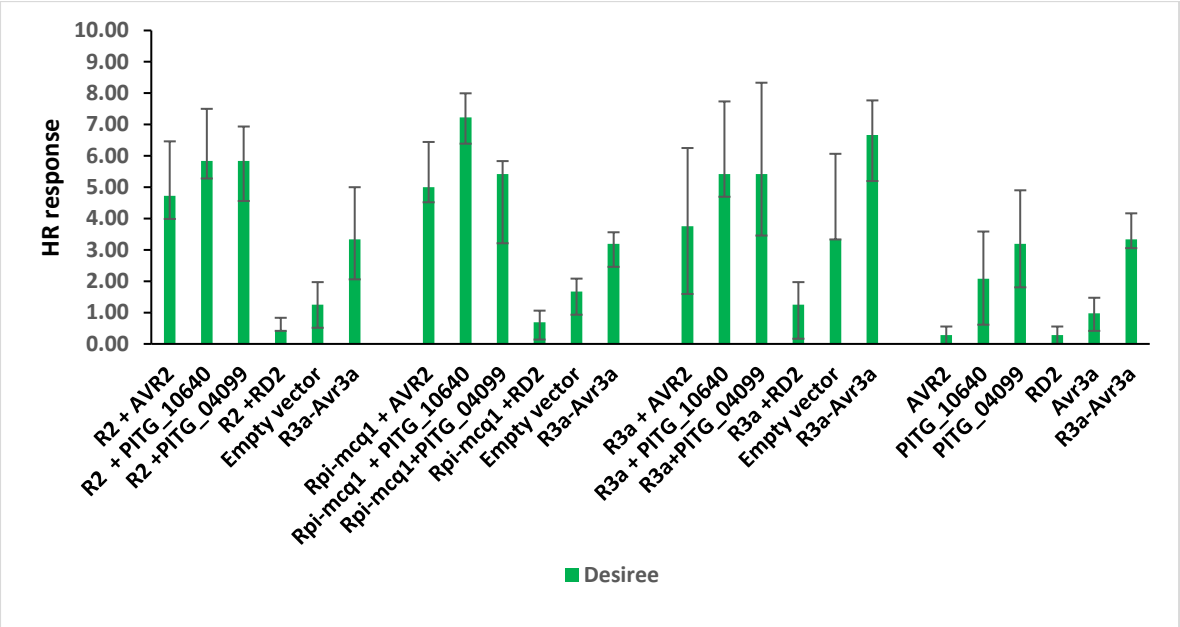


Figure 8. Co-infiltration of *R2*, *Rpi-mcq1* and *R3a* with different effectors in potato cultivar Désirée showing the HR responses.

3.2 Single infiltration response of *Désirée-R2, Désirée-Rpi-mcq1* and wild species

To determine the recognition pattern of AVR2, PITG_10640, PITG_04099 and RD2 effectors by *R2* and *Rpi-mcq1*, Single infiltration was done in Désirée-*R2*, Désirée-*Rpi-mcq1* plants and wild species. The wild species were tested because if any effector was recognized by the transformant then, of course, it will be recognized by the donor wild species. The potato plants were infiltrated with the effectors and plants were scored for the occurrence of cell death responses after five days of infiltration. The AVR2, PITG_10640 and PITG_04099 were recognized by Désirée-*R2*, Désirée-*Rpi-mcq1*, Hjt349-3 and Edn151-1 plants with either high or low level of HR response (figure 9). AVR2 and PITG_10640 were recognized by Mcq717-3, whereas only AVR2 was recognized by the Hcb353-8 plants. It was also found that some plants responded to the negative control, empty vector as well with a low HR score. It indicates the sensitivity to *Agrobacterium*. As expected, the positive control *R3a-Avr3a* also provided a positive cell death response in all plants. The results are similar with the result of potato cultivar Bintje and Désirée. The HR index score and the HR pictures are presented in appendix 2 and 4, respectively.

The overall result of agroinfiltration confirms that the candidate effectors AVR2, PITG_10640, PITG_04099 cause cell death response in Désirée- *R2*, Désirée- *Rpi-mcq1*.

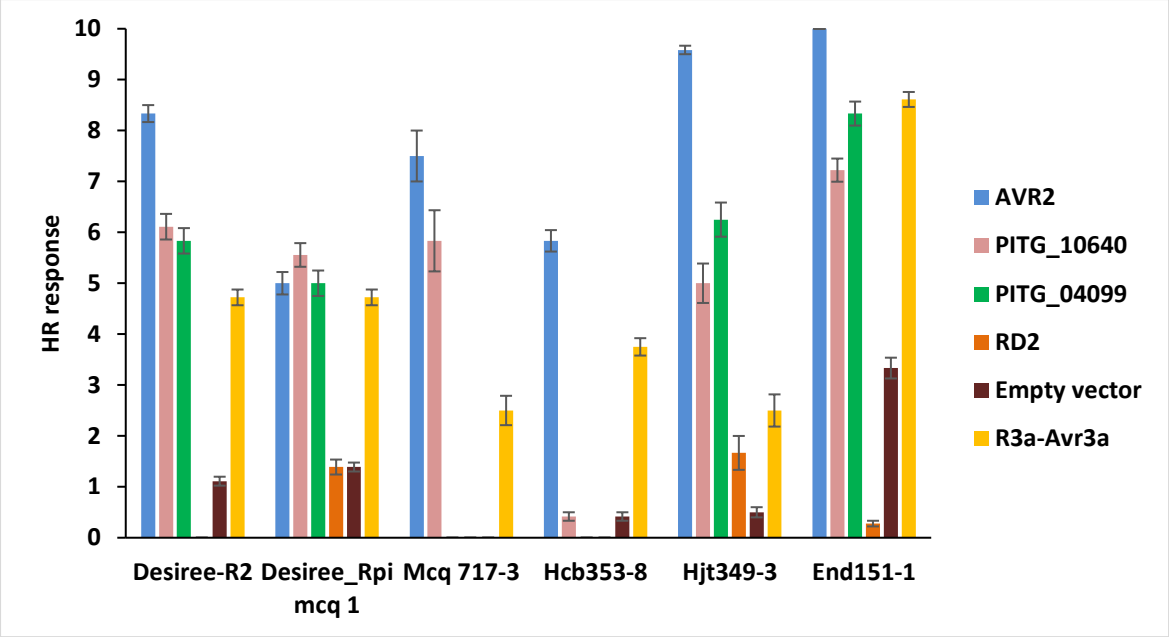


Figure 9. Agro-infiltration cell death response of *Désirée-R2, Désirée-Rpi-mcq1* and wild species

3.3. Virulence profile of *P. infestans* isolates

To know the virulence profile of the *P. infestans* isolates from Peru (Peru 4, Peru 29 and Peru 42) on Désirée-*R2*, Désirée-*Rpi-mcq1* and Désirée-*Rpi-hcb,* A detached leaf assay (DLA) was conducted. Eleven different genotypes (donor of *R* gene, transformant and Désirée control) were successfully spot inoculated with different isolates of *P. infestans*. The isolate Peru 29 was very slow growing and no lesion was developed in any genotype (data not shown). The infection efficiency (IE) and lesion growth rate (LGR) was calculated from the infected leaf. The isolate Peru 4 showed no infection on the detached leaf of Désirée-*R2*, Désirée-*Rpi-mcq1,* Mcq-717-3, Hcb353-8, Double-2, Double-4 and Snk213-1. The infection efficiency was higher in the control plant Désirée and Désirée-*Rpi-hcb1.2- 56* than the others. The lesion growth rate (LGR) was not significantly different among the infected genotypes. On the other hand, the isolate Peru 42 showed higher infection efficiency in double 2, double 4, Désirée-*Rpi-hcb1.1-22,* Désirée-*Rpi-hcb1.2-3,* Désirée-*Rpi-hcb1.2-56* and Désirée, which is similar with the result of Peru 4, except for Double 2 and 4. The lesion expansion was also higher in double 4, Désirée-*Rpi-hcb1.2-3* and Désirée which was different from the others (figure 10).

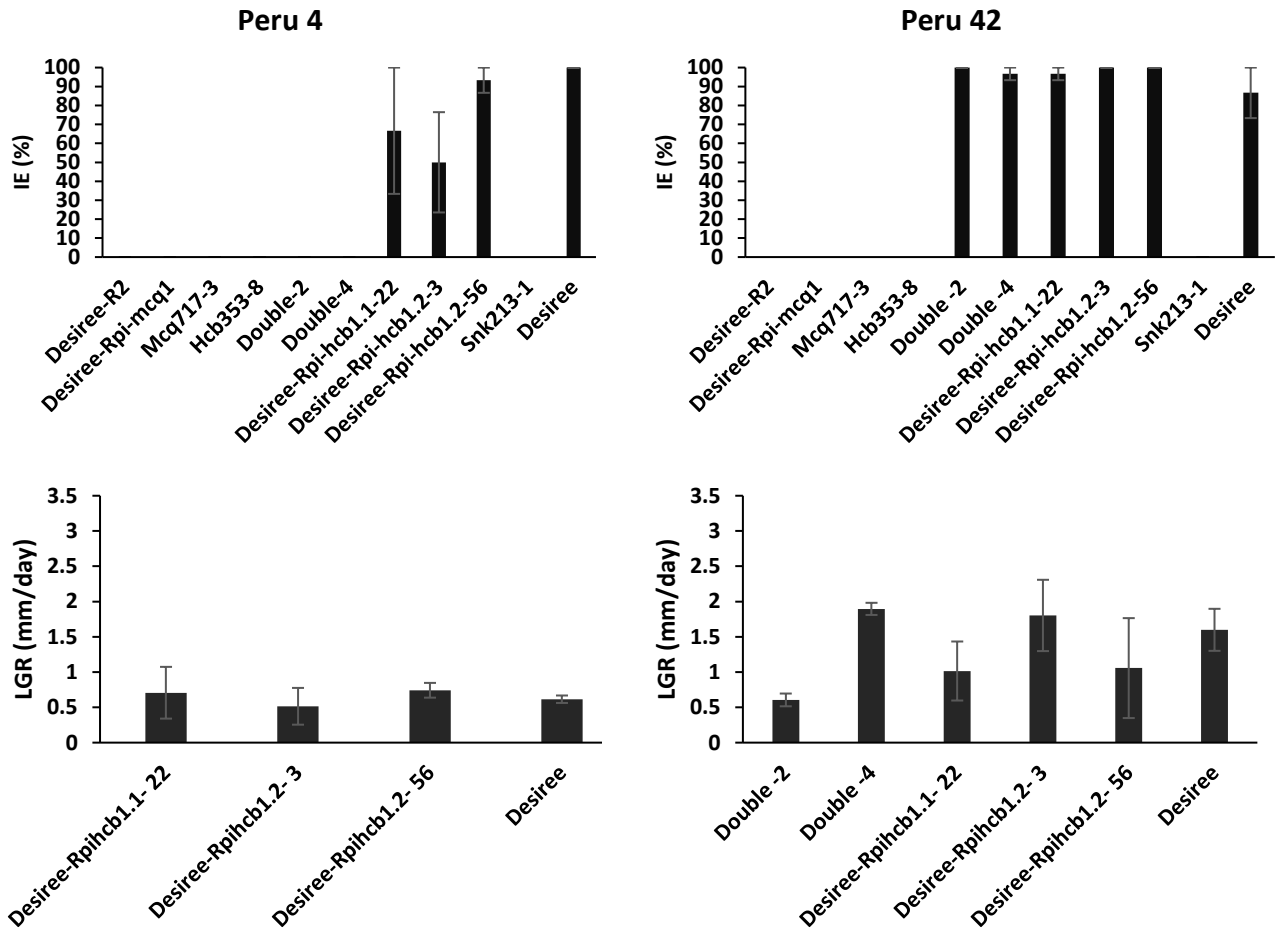


Figure 10. Infection efficiencies (IE) and lesion growth rates (LGRs) of Peru 4 and Peru 42 *P. infestans* isolates on *R2*, *Rpi-mcq1*, *Rpi-hcb* (transformants and donor) and Désirée control plants.

All the eleven different genotypes were also tested with PIC 99183 and IPO-0 of *P. infestans* isolates in separate inoculation experimnet. The isolate PIC 99183 developed lesions in Double 4 and Désirée. The Highest infection efficiency was in the transformant double 4 (figure 11). This result is similar to the result of lesion growth rate. The isolate IPO-0 performed the highest infection efficiency and lesion growth rate in Désirée plants.

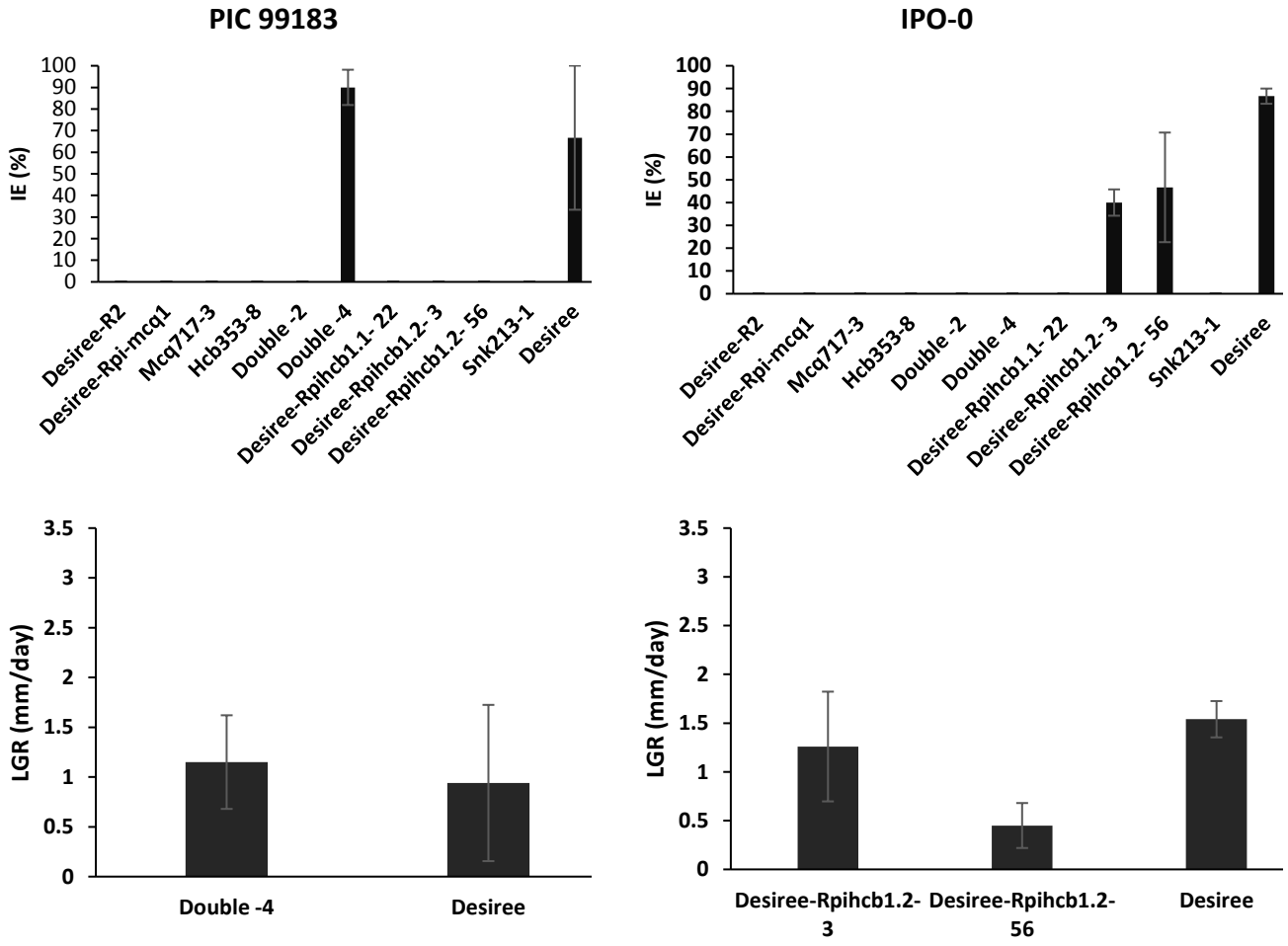


Figure 11. Infection efficiencies (IE) and lesion growth rates (LGRs) of PIC 99183 and IPO-0 *P. infestans* isolates on *R2*, *Rpi-mcq1*, *Rpi-hcb* (transformants and donor) and Désirée control plants.

The genotypes were also tested with the differential isolates NL07434 and NL09096 of *P. infestans*. The isolate NL07434 was avirulent to Désirée-*R2*, but it was virulent to Désirée-*Rpi-mcq-1.* It doesn't establish any lesion in Désirée-*R2* and Hcb-353-8. The lesion growth rate was also different among the infected genotypes. The highest lesion growth rate was found in Désirée-*Rpi-hcb1.2- 56* (figure 12). The isolate NL09096 also showed a similar type of result for the infection efficiency and lesion growth rate.

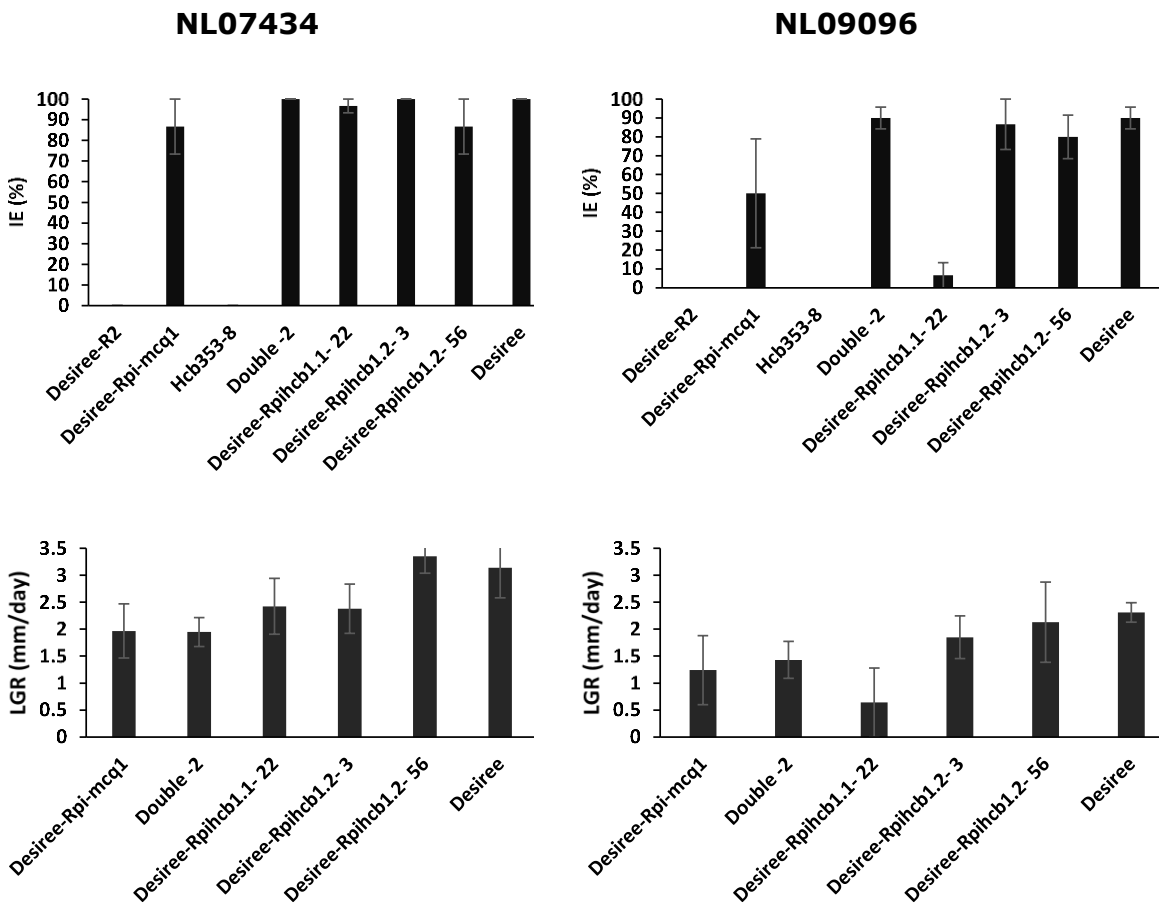


Figure 12. Infection efficiencies (IE) and lesion growth rates (LGRs) of NL07434 and NL09096 *P. infestans* isolates on *R2*, *Rpi-mcq1*, *Rpi-hcb* (transformants and donor) and Désirée control plants.

The sporulation outlook of the inoculated genotypes for the isolates was also observed. The sporulation was started on the fifth day of inoculation. Initially, small black lesions were developed, but later became larger with spores. Désirée plant leaves responded with big lesions and spores with all the isolates. The pictures of inoculated leaves are presented in the appendix 6. The sporulation pattern is similar with the infection of potato leaves, means high infection efficiency leads to more sporulation.

Table 7. The sporulation pattern of different *P. infestans* isolates over different genotypes

Genotypes	**Peru-4**	**Peru 42**	**IPO-0**	**PIC99183**	**NL07434**	**NL09096**
Désirée-*R2*	NS	NS	NS	NS	NS	NS
Désirée-*Rpi-mcq1*	NS	NS	NS	NS	S	S
Mcq717-3	NS	NS	NS	NS	*	*
Hcb353-8	NS	NS	NS	NS	NS	NS
Double transformant- 2	NS	S	NS	NS	S	S
Double transformant- 4	NS	S	NS	S	*	*
Désirée-*Rpi-hcb1.1* transformant-22	S	S	NS	NS	S	S
Désirée-*Rpi-hcb1.2* transformant-3	S	S	S	NS	S	S
Désirée-*Rpi-hcb1.2* transformant-56	S	S	S	NS	S	S
Snk-213-1	NS	NS	NS	NS	*	*
Désirée	S	S	S	S	S	S

NS means Non-sporulation, S means Sporulation and * indicates missing

4. Discussion

In this chapter, the results of agro-infiltration and detach leaf assay will be discussed.

4.1 Co-agroinfiltration response in *N. benthamiana*, Bintje and Désirée

Two *R* genes**,** *R2* and *Rpi-mcq1* were isolated from wild *Solanum* species and showed a differential disease resistant to *P. infestans* isolates of NL07434, NL12003 and NL09096. These two genes showed a similar resistance pattern with all other isolates tested (Aguilera-Galvez et al., Submitted). To find out the reason for this different resistance pattern more investigation was carried out. Some new effectors, diverse allelic variants of AVR2 or new variants of the AVR2 family may be the reason for this difference, that's why *Agrobacterium*-mediated transient transformation (ATTA) was carried out in *N. benthamiana*, Bintje and Désirée with three effectors that were upregulated after infection with NL07434 isolated and could be differentially recognized by *R2* or *Rpi-mcq1*. Surprisingly, there was no response in co- agroinfiltration in *N. benthamiana* for any effector, even in co-infiltrations with R2 and AVR2 combinations that have been confirmed several times before this thesis. No simple justification can be given for this difference in result. We have tried several times with different optimization of the procedure. We changed the OD, culture, media and the stock of *R* gene and effectors, but it doesn't work. Sometimes, only the positive control showed some HR responses. It might be the seasonal difference, because it is difficult to provide a constant environmental condition to greenhouse all over the year. The humidity and temperature are easy to control in greenhouse but it is difficult to provide a precise light condition in all season. Light plays an important role in cell death response and modulates ETI (Gao et al., 2017). The experiment was conducted in winter season and the light condition might be a reason of this.

In potato plants, Bintje and Désirée, co-agroinfiltrations of *R2 and Rpi-mcq1* revealed HR responses with the effectors AVR2, PITG_10640 and PITG_04099. The effector RD2 was not recognized by any *R* genes. However, the *R* gene *R3a* also recognizes the effectors in co-infiltration with potato cultivar Bintje and Désirée. *R3a* is from different *R* gene family of *Solanum* species and specifically recognizes Avr3a. That means the recognition of the new effectors tested is nonspecific to *R2* or *Rpi-mcq1* and therefore it cannot explain the differential virulence profile. Additionally, in previous experiments *R3a* did not show HR responses in co-agroinfiltration with AVR2, it indicates something happened in the experiment and should be repeated to confirm the recognition of the mentioned effectors.

4.2 Single infiltration response in *Désirée-R2, Désirée-Rpi-mcq1* and wild species

To confirm the result of the co-infiltration test, the same effectors were tested with the potato variety that contained different *Rpi* genes. The wild donor species were also tested

to confirm the result. The effector AVR2, PITG_10640 and PITG_04099 were recognized by Désirée-*R2* and its wild donor plant (Hjt349-3 and Edn151-1). On the other hand, the same effectors AVR2, PITG_10640 and PITG_04099 were recognized by Désirée-*Rpi-mcq1* and its wild donor plant Mcq717-3 recognizes AVR2 and PITG_10640 only. Another donor plant of *Rpi-hcb* gene only recognizes the effector AVR2. In all plant, the positive control provided cell death response was expected. This result is similar with the result of co-agroinfiltration.

As we know that, Désirée- *R2* and Désirée-*Rpi-mcq1* showed the different result with *P. infestans* isolates (Table 1). Their recognition of AVR2 family members was also different (Table 2). It indicates that the diverse pattern observed in disease test could be explained by the differential recognition of other variant of AVR2 effector family. Chizhik & Martynov (2017) identified seven variants of AVR2 from *P. infestans* isolates of Moskow, that will be interesting to test. In our experiments, both Désirée-*R2* and Désirée-*Rpi-mcq1* recognizes the effector AVR2, PITG_10640 and PITG_04099. So, the difference in disease test result (Table 1) was not also for those effectors. We have to find out one effector which is specifically recognized by *R2* plants, but not by *Rpi-mcq1* plants.

In this experiment, the genotype Snk-213-1 was completely failed. It always developed HR in all infiltration site. The genotype Hcb, Hjt and Snk were difficult to infiltrate, because of the leaf size. Expert personnel are necessary to infiltrate those genotypes without any leaf injury. In ATTA test of the potato plant, sometimes it was observed that the plants responded with the empty vector. It was happened due to the sensitivity of *Agrobacterium*. The compatibility of *Agrobacterium* and plant species plays an important rule for ATTA (Goodin et al., 2015). Sometimes they are virulent for the plant species and causes cell death and necrosis of the leaf. The optical density (OD) may also influence the response of the plant, the age of plant is also an important factor. These factors depend on the *R* gene and the plant species. So, optimization in ATTA is necessary for a successful result.

4.3 *Phytophthora infestans* isolates and disease test

There are many methods that have been developed to evaluate the virulence profile of *Phytophthora infestans* isolates, including whole-plant greenhouse assays (Stewart et al., 1983), field test and detached leaf assay (Vleeshouwers et al., 1999). Among them, the detached leaf assay is one of the reliable and relatively fast methods for determination of virulence pattern of isolates and quantification of resistance level of the genotypes. Another important advantage of is to escape the infestation of other diseases, like powdery mildew, which is a common problem in greenhouse assays.

In this research, our interest was to know about the virulence profile of *P. infestans* isolates from Peru on Désirée-*R2*, Désirée-*Rpi-mcq1* and Désirée-*Rpi-hcb*. As we know that, the *R2* and *Rpi-mcq1* are resistant to *P. infestans* isolates, and were cloned from *Solanum* of Mexico and Peru, respectively. *Rpi-mcq1* was cloned from *S. mochiquense*, a Peruvian wild

potato species of south America (Smilde et al., 2005). Since these two genes were from two different geographic regions, that's why we hypothesized that the wild *Solanum* from Peru (as *S. mochiquense and S. huancabambense*) and *Rpi-mcq1* gene will evolve with the effectors *P. infestans* isolates from the same geographic area. Three isolates Peru 4, Peru 29 and Peru 42 were tested with eleven genotypes. The isolates Peru 29 was very slow growing and maybe it needs more time and different growth condition for germination. The other two isolates Peru 4 and Peru 42 grew very well and made infections. Both isolates were recognized by the *Rpi-mcq1* and the donor of this gene Mcq-717-3 and donor of *Rpi-hcb* (Hcb-353-8). But the interesting thing is that it was not recognized by the *Désirée-Rpi-hcb* transformants. The possible reason could be the resistant gene was a weak *R* gene. Similar results also found in double transformant for the isolate Peru 42, stacked genes were nonfunctional or weak. But in Peru 4, the double transformants were free from infection. It specifies that the stacking may cause some changes in the plant that alters the growth and resistance, or the infection can be happened by chance. Interestingly, *R2* gene was also avirulent to both isolates which confirms that these two isolates are not suitable for the study of the differential interaction between *R2* and *Rpi-mcq1.* However, based on the infection efficiency and lesion growth rate the isolate Peru 42 is more aggressive than the Peru 4.

The Peruvian and Bolivian Andes are thought to be the centre of diversity of potato, and the area is rich in biodiversity of numerous *solanaceous* crops which are the hosts of *P. infestans* (Perez et al., 2001). This high host diversity may affect the pathogen's population structure and evolution. The area where diverse potato varieties are still cultivated, it might be expected the high pathogen diversity in that area (Wolfe and Finckh,1997). Forbes et al. (1997) found that there is a risk of sexual reproduction between the border site pathogen of Peru and Bolivia. Because, *P. infestans* population is A1 mating type in peru, but A2 mating type (Goodwin et al., 1994) in Bolivia. This recombination and migration could lead to enhance the risks for the potato grower and plant genetic resources of Andes area. However, testing of those genotypes with more isolates from Peru will help to know the virulence profile of the *Rpi* gene containing transformants.

The disease test was also performed in all the genotypes with the isolate PIC99183 and IPO-0 of *P. infestans*. Potatoes with either *Rpi* gene, *R2* or *Rpi-mcq1,* were resistant against the isolates. The result is consistent with the result of the previous experiment of this project. The isolate PIC99183 was avirulent to Désirée-*Rpi-hcb* transformants but IPO-0 was virulent to Désirée-*Rpi-hcb* transformants. The Désirée control plant was the susceptible plant for all isolates.

Additionally, with this experiment two isolates, NL07434 and NL09096 were also included to validate the result of previous experiments result. In this experiment, we also found the similar result with the previous experiment where the isolate NL07434 was avirulent to Désirée-*R2*, but it was virulent to Désirée-*Rpi-mcq-1. Rpi-hcb* transformants were

susceptible to both isolates, but their donor species Hcb-353-8 was free from any infection. It might be possible that there is another gene which confers resistant in the donor species or the transformants contains a weak gene or the transform gene was not functional properly.

Pathogen aggressiveness is determined by infection efficiency, spore production rate, infectious period, latent period and lesion size (Pariaud et al., 2009). At the population level, it is determined by the pathogen sporulation behaviour. If a pathogen sporulates more bounteously in a population, it gets more scope to establish its genes in the gene pool of the population (Lebreton et al., 1999; Suassuna et al., 2004). More the sporulation more will be the susceptibility. According to the result of disease test, the variety Désirée showed sporulation upon infection of all tested *P. infestans* isolates, because it doesn't have the resistant gene to combat the isolates. Based on the sporulation behaviour of the Peruvian isolates, Peru 42 was more aggressive than the Peru 4.

The virulence profile of new isolates is necessary to know the level of resistance and susceptibility of the genotypes. Detach leaf assay is considered as a representative of testing resistant gene functionality. It provides the result of spore infection which can be comparable with the field disease test result of the living plant (Vleeshouwers et al., 1999). The result of DLA is more reliable than the ATTA because sometimes control Désirée plant shows HR in ATTA without possessing any *R* gene. On the other and, the aggressiveness and virulence of the pathogen isolates can change the durability of resistance in the plant (Flier et al., 2001).

5. Conclusion and Recommendations

The aim of the thesis was to find out the reason of differential disease resistance of *R2* and *Rpi-mcq1* with some isolates of *P. infestans*. To find out the reason, *Agrobacterium-*

mediated transient transformation was carried out in *N. benthamiana* and potato plants with different effectors. There was no response in *N. benthamiana*, repetition of this effector test in *N. benthamiana* is recommended. The *R3a* gene showed HR with AVR2, so this experiment on Désirée and Bintje should be repeated. Both *R2* and *Rpi-mcq1* recognize the effectors but not differentially. So, further screening is necessary with other effectors that will recognize the two *R* gene differentially. Since the isolates NL07434, NL12003 and NL09096 provided different result in disease test, can be a source for new effector test. The upregulated effectors from RNA-seq data of those isolates, specially the common effectors in three isolates are recommended for further test. Also, it will be interesting to test the new AVR2 variants found in the isolates from Moscow. We can test the expression of those variants after infection of potato with our differential isolates.

Another aim of the thesis was to see the virulence profile of the *P. infestans* isolates from Peru. The *Rpi-mcq1* gene was collected from Peru and it showed resistance against the isolates of same geographic location. But the other gene *R2* also showed resistance against the isolates from Peru. The isolate Peru 29 was slow growing and it should be tested with the genotypes in a future experiment. In future, more isolates should be included in the DLA test to find out a differential isolate which will be avirulent to *Rpi-mcq1* but virulent to *R2*. It will help to find out the resistant spectra of double transformants.

6. Acknowledgement

At the inception, I would like to acknowledge my minor thesis supervisor Dr. Vivianne Vleeshouwers for her guidance and giving me the opportunity to do my MSc minor thesis in a nice, time demanding topic. This research work was a part of PhD project of Carolina Aguilera Galvez and she was my daily supervisor. I would like to extend my profound

gratitude, vast appreciation to Carolina Aguilera Galvez for her support, help, guidance and daily supervision. During the full four months, I got the opportunity to learn a lot of new things from her. I wish every success of her life and a bright future.

I would like to thank Dr. Evert Jacobsen for being an examiner of my thesis. I would also like to thank the other group members of resistance breeding Solanaceae group. I especially would like to thank Doret Wouters and Gert van arkel for helping me in lab work, and Trudy van den Bosch for retrieve from Liquid Nitrogen the Peruvian isolates. I express my gratitude and thanks to Happyka Fillianti and Jaap Wolters who helped me a lot during the thesis period.

Finally, I must express my special appreciation and indebtedness to my parents who are always inspiring and supporting me for my higher study. I am grateful to my life partner for her love, care, sacrifice and encouragement to complete the Master degree. I have a deep sense of gratitude for the Dutch government and all the people engaged in the selection of Netherland Fellowship Program (NFP) for giving me the scholarship opportunity. I am grateful to my job institute, Bangladesh Agricultural Research Institute for providing me two years long study leave to take part in this MSc program.

7. References

Anderson, R. G., Deb, D., Fedkenheuer, K., & McDowell, J. M. (2015). Recent progress in RXLR effector research. *Molecular Plant-microbe Interactions*, *28*(10), 1063-1072. https://doi.org/ 10.1094/ MPMI-01-15-0022-CR.

Birch, P. R., Bryan, G., Fenton, B., Gilroy, E. M., Hein, I., Jones, J. T., ... & Toth, I. K. (2012). Crops that feed the world 8: Potato: are the trends of increased global production sustainable?. *Food Security*, *4*(4), 477-508. https://doi.org/10.1007/s12571-012-0220-1.

Chizhik, V. K., & Martynov, V. V. (2017). Polymorphism of the Avr2 gene of oomycete Phytophthora infestans (Mont.) de Bary in the population of Moscow region. *Russian Journal of Genetics*, *53*(12), 1328-1334. https://doi.org/10.1134/S1022795417120031.

Dodds, P. N., & Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics*, *11*(8), 539-548. https://doi.org/10.1038/nrg2812.

Dou, D., Kale, S. D., Wang, X., Jiang, R. H., Bruce, N. A., Arredondo, F. D., ... & Tyler, B. M. (2008). RXLR-mediated entry of Phytophthora sojae effector Avr1b into soybean cells does not require pathogen-encoded machinery. *The Plant Cell*, *20*(7), 1930-1947. https://doi.org/10.1105/ tpc.107.056093.

Du, J. (2014). *Elicitin-triggerd apoplastic immunity against late blight in potato*. Wageningen University.

Flier, W. G., Turkensteen, L. J., Van Den Bosch, G. B. M., Vereijken, P. F. G., & Mulder, A. (2001). Differential interaction of Phytophthora infestans on tubers of potato cultivars with different levels of blight resistance. *Plant Pathology*, *50*(3), 292-301. https://doi.org/10.1046/j.1365-3059.2001.00574.x.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annual review of phytopathology*, *9*(1), 275-296.

Foster, S. J., Park, T. H., Pel, M., Brigneti, G., Śliwka, J., Jagger, L., ... & Jones, J. D. (2009). Rpi-vnt1. 1, a Tm-22 homolog from Solanum venturii, confers resistance to potato late blight. *Molecular plant-microbe interactions*, *22*(5), 589-600. https://doi.org/10.1094/MPMI-22-5-0589.

Fry, W. (2008). Phytophthora infestans: the plant (and R gene) destroyer. *Molecular plant pathology*, *9*(3), 385-402. https://doi.org/10.1111/j.1364-3703.2007.00465.x

Fry, W. E., Cooke, D. E., McLeod, A., Forbes, G. A., & Cao, K. (2008). Population genetics and population diversity of Phytophthora infestans. *Oomycete genetics and genomics: diversity, interactions, and research tools*, 139-164. https://doi.org/10.1002/9780470475898.ch7.

Gao, Y., Wu, Y., Du, J., Zhan, Y., Sun, D., Zhao, J., ... & He, K. (2017). Both Light-Induced SA Accumulation and ETI Mediators Contribute to the Cell Death Regulated by BAK1 and BKK1. *Frontiers in plant science*, *8*. https://doi.org/10.3389/fpls.2017.00622.

Gebhardt, C. (1994). RFLP mapping in potato of qualitative and quantitative genetic loci conferring resistance to potato pathogens. *American Journal of Potato Research*, *71*(5), 339-345. https://doi.org/10.1007/BF02849060.

Gómez-Alpizar, L., Carbone, I., & Ristaino, J. B. (2007). An Andean origin of Phytophthora infestans inferred from mitochondrial and nuclear gene genealogies. *Proceedings of the National Academy of Sciences*, *104*(9), 3306-3311. https://doi.org/10.1073/pnas.0611479104.

Goodin, M. M., Zaitlin, D., Naidu, R. A., & Lommel, S. A. (2015). Nicotiana benthamiana: Its History and Future as a Model for Plant-Pathogen Interactions. *Molecular plant-microbe interactions: MPMI*, *2015*(1), 28-39. https://doi.org/10.1094/MPMI-00-00-1015-REV.testissue

Goodwin, S. B., Cohen, B. A., & Fry, W. E. (1994). Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. *Proceedings of the National Academy of Sciences*, *91*(24), 11591-11595.

Goodwin, S. B., Cohen, B. A., & Fry, W. E. (1994). Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. *Proceedings of the National Academy of Sciences*, *91*(24), 11591-11595.

Grünwald, N. J., & Flier, W. G. (2005). The biology of Phytophthora infestans at its center of origin. *Annu. Rev. Phytopathol.*, *43*, 171190. https://doi.org/10.1146/annurev.phyto.43.040204.135906.

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H., Handsaker, R. E., Cano, L. M., ... & Bozkurt, T. O. (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature*, *461*(7262), 393-398. http://dx.doi.org/10.1038/nature08358.

Haverkort, A. J., Struik, P. C., Visser, R. G. F., & Jacobsen, E. (2009). Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. *Potato Research*, *52*(3), 249-264. https://doi.org/10.1007/s11540-009-9136-3.

Hein, I., Gilroy, E. M., Armstrong, M. R., & Birch, P. R. (2009). The zig-zag-zig in oomycete–plant interactions. *Molecular Plant Pathology*, *10*(4), 547-562. https://doi.org/10.1111/j.1364-3703.2009.00547.x.

Jo, K. R., Kim, C. J., Kim, S. J., Kim, T. Y., Bergervoet, M., Jongsma, M. A., ... & Vossen, J. H. (2014). Development of late blight resistant potatoes by cisgene stacking. *BMC biotechnology*, *14*(1), 50. https://doi.org/10.1186/1472-6750-14-50.

Jones, J. D., Witek, K., Verweij, W., Jupe, F., Cooke, D., Dorling, S., ... & Foster, S. (2014). Elevating crop disease resistance with cloned genes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1639), 20130087. https://doi.org/10.1098/rstb.2013.0087.

Kamoun, S. (2006). A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual review of phytopathology*, *44*. https://doi.org/10.1146/annurev.phyto.44.070505.143436.

Kim, H. J., Lee, H. R., Jo, K. R., Mortazavian, S. M., Huigen, D. J., Evenhuis, B., ... & Vossen, J. H. (2012). Broad spectrum late blight resistance in potato differential set plants MaR8 and MaR9 is conferred by multiple stacked R genes. *Theoretical and applied genetics*, *124*(5), 923-935. https://doi.org/10.1007/s00122-011-1757-7.

Lebreton, L., Lucas, J. M., & Andrivon, D. (1999). Aggressiveness and competitive fitness of Phytophthora infestans isolates collected from potato and tomato in France. *Phytopathology*, *89*(8), 679-686. https://doi.org/10.1094/PHYTO.1999.89.8.679.

Li, Y., de Vries, R., Slaghek, T., Timmermans, J., Cohen Stuart, M. A., & Norde, W. (2009). Preparation and characterization of oxidized starch polymer microgels for encapsulation and controlled release of functional ingredients. *Biomacromolecules*, *10*(7), 1931-1938. https://doi.org/10.1021/bm900337n.

Macho, A. P., & Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Molecular cell*, *54*(2), 263-272. https://doi.org/10.1016/j.molcel.2014.03.028.

Malcolmson, J. F., & Black, W. (1966). New R genes in Solanum demissum Lindl. and their complementary races of Phytophthora infestans (Mont.) de Bary. *Euphytica*, *15*(2), 199-203. https://doi.org/10.1007/BF00022324.

McDonald, B. A., & Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annual review of phytopathology*, *40*(1), 349-379. https://doi.org/10.1146/ annurev.phyto.40.120501.101443.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Niks, R. E., Parlevliet, J. E., Lindhout, P., & Bai, Z. (2011). Breeding crops with resistance to diseases and pests (p. 198). *The Netherlands: Wageningen Academic Publishers*.

Pariaud, B., Ravigné, V., Halkett, F., Goyeau, H., Carlier, J., & Lannou, C. (2009). Aggressiveness and its role in the adaptation of plant pathogens. *Plant Pathology*, *58*(3), 409-424. https://doi.org/10.1111/j.1365-3059.2009.02039.x

Perez, W. G., Gamboa, J. S., Falcon, Y. V., Coca, M., Raymundo, R. M., & Nelson, R. J. (2001). Genetic structure of Peruvian populations of Phytophthora infestans. *Phytopathology*, *91*(10), 956-965. https://doi.org/10.1094/PHYTO.2001.91.10.956.

Raffaele, S., Farrer, R. A., Cano, L. M., Studholme, D. J., MacLean, D., Thines, M., ... & Meyers, B. C. (2010). Genome evolution following host jumps in the Irish potato famine pathogen lineage. *Science*, *330*(6010), 1540-1543. https://doi.org/10.1126/science.1193070.

Rauscher, G. M., Smart, C. D., Simko, I., Bonierbale, M., Mayton, H., Greenland, A., & Fry, W. E. (2006). Characterization and mapping of Rpi-ber, a novel potato late blight resistance gene from Solanum berthaultii. *Theoretical and applied genetics*, *112*(4), 674-687. https://doi.org/ 10.1007/ s00122-005-0171-4.

Saunders, D. G., Breen, S., Win, J., Schornack, S., Hein, I., Bozkurt, T. O., ... & Kamoun, S. (2012). Host protein BSL1 associates with Phytophthora infestans RXLR effector AVR2 and the Solanum demissum immune receptor R2 to mediate disease resistance. *The Plant Cell*, *24*(8), 3420-3434. DOI: https://doi.org/10.1105/tpc.112.099861.

Smilde, W. D., Brigneti, G., Jagger, L., Perkins, S., & Jones, J. D. G. (2005). Solanum mochiquense chromosome IX carries a novel late blight resistance gene Rpi-moc1. *Theoretical and Applied Genetics*, *110*(2), 252-258. https://doi.org/10.1007/s00122-004-1820-8.

Stewart, H. E., Flavelle, P. H., McCalmont, D. C., & Wastie, R. L. (1983). Correlation between glasshouse and field tests for resistance to foliage blight caused by Phytophthora infestans. *Potato Research*, *26*(1), 41-48. https://doi.org/10.1007/BF02357372.

Suassuna, N. D., Maffia, L. A., & Mizubuti, E. S. G. (2004). Aggressiveness and host specificity of Brazilian isolates of Phytophthora infestans. *Plant Pathology*, *53*(4), 405-413. https://doi.org/10.1111/j.1365-3059.2004.01043.x.

Tan, M. A., Hutten, R. C., Celis, C., Park, T. H., Niks, R. E., Visser, R. G., & van Eck, H. J. (2008). The RPi-mcd1 locus from Solanum microdontum involved in resistance to Phytophthora infestans, causing a delay in infection, maps on potato chromosome 4 in a cluster of NBS-LRR genes. *Molecular Plant-Microbe Interactions*, *21*(7), 909-918. https://doi.org/10.1094/MPMI-21-7-0909.

Vleeshouwers, V. G., Raffaele, S., Vossen, J. H., Champouret, N., Oliva, R., Segretin, M. E., ... & Pel, M. A. (2011). Understanding and exploiting late blight resistance in the age of effectors. *Annual review of phytopathology*, *49*, 507-531. https://doi.org/10.1146/annurev-phyto-072910-095326.

Vleeshouwers, V. G., van Dooijeweert, W., Keizer, L. P., Sijpkes, L., Govers, F., & Colon, L. T. (1999). A laboratory assay for Phytophthora infestans resistance in various Solanum species reflects the field situation. *European journal of plant pathology*, *105*(3), 241-250. http://doi.org /10.1023/A:1008710700363.

Vossen, E. A., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., ... & Allefs, S. (2005). The Rpi-blb2 gene from Solanum bulbocastanum is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato. *The Plant Journal*, *44*(2), 208-222. https://doi.org/10.1111/j.1365-313X.2005.02527.x.

Wang, S., Boevink, P. C., Welsh, L., Zhang, R., Whisson, S. C., & Birch, P. R. (2017). Delivery of cytoplasmic and apoplastic effectors from Phytophthora infestans haustoria by distinct secretion pathways. *New Phytologist*, *216*(1), 205-215. https://doi.org/10.1111/nph.14696.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., ... & Hein, I. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature*, *450*(7166), 115-118. https://doi.org/10.1038/nature06203.

Whisson, S. C., Boevink, P. C., Wang, S., & Birch, P. R. (2016). The cell biology of late blight disease. *Current opinion in microbiology*, *34*, 127-135. https://doi.org/10.1016/ j.mib.2016. 09.002.

Win, J., Chaparro-Garcia, A., Belhaj, K., Saunders, D. G. O., Yoshida, K., Dong, S., ... & Kamoun, S. (2012, January). Effector biology of plant-associated organisms: concepts and perspectives.

In *Cold Spring Harbor symposia on quantitative biology* (Vol. 77, pp. 235-247). Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/sqb.2012.77.015933.

Witek, K., Jupe, F., Witek, A. I., Baker, D., Clark, M. D., & Jones, J. D. (2016). Accelerated cloning of a potato late blight-resistance gene using RenSeq and SMRT sequencing. *Nature biotechnology*, *34*(6), 656-660 https://doi.org/10.1038/nbt.3540.

Wolfe, M. S., & Finckh, M. R. (1997). Diversity of host resistance within the crop: effects on host, pathogen and disease. *Resistance of crop plants against fungi*.

Zhu, S., Li, Y., Vossen, J. H., Visser, R. G., & Jacobsen, E. (2012). Functional stacking of three resistance genes against Phytophthora infestans in potato. *Transgenic research*, *21*(1), 89-99. https://doi.org/10.1007/s11248-011-9510-1.

Appendices

In the appendix chapter, the additional information which was not shown in the other chapter of thesis will be presented.

Appendix 1. HR response index of Bintje and Désirée in Co-agroinfiltration assay

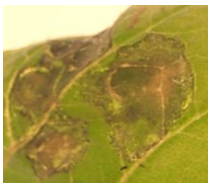
R + AVR pair	Désirée	Bintje
R2 + AVR2	4.72	2.22
R2 + PITG_10640	5.83	3.89
R2 +PITG_04099	5.83	2.50
R2 +RD2	0.42	0.00
Negative control	1.25	1.11
R3a-Avr3a	3.33	3.33
Rpi-mcq1 + AVR2	5.00	3.33
Rpi-mcq1 + PITG_10640	7.22	3.33
Rpi-mcq1+PITG_04099	5.42	4.17
Rpi-mcq1 +RD2	0.69	1.11
Negative control	1.67	1.11
R3a-Avr3a	3.19	3.06
R3a + AVR2	3.75	3.19
R3a + PITG_10640	5.42	8.75
R3a+PITG_04099	5.42	5.14
R3a +RD2	1.25	1.81
Negative control	3.33	2.50
R3a-Avr3a	6.67	7.22
AVR2	0.28	0.83
PITG_10640	2.08	2.22
PITG_04099	3.19	1.39
RD2	0.28	0.28
Avr3a	0.97	0.56
R3a-Avr3a	3.33	2.73

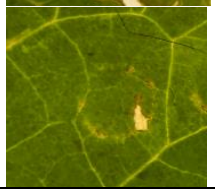
Appendix 2. HR response index of *Désirée-R2, Désirée-Rpi-mcq1* and wild species in Agroinfiltration assay

Effectors	*Désirée-R2*	*Désirée-Rpi-mcq 1*	Mcq 717-3	Hcb353-8	Hjt349-3	End151-1
AVR2	8.3	5.0	7.5	5.8	9.6	10.0
PITG_10640	6.1	5.6	5.8	0.4	5.0	7.2
PITG_04099	5.8	5.0	0.0	0.0	6.3	8.3
RD2	0.0	1.4	0.0	0.0	1.7	0.3
Empty vector	1.1	1.4	0.0	0.4	0.5	3.3
R3a-Avr3a	4.7	4.7	2.5	3.8	2.5	8.6

Appendix 3. Supplementary pictures showing the HR responses to different effectors by *R2* and *Rpi-mcq1.1* upon agro-coinfiltrations in Désirée and Bintje, respectively

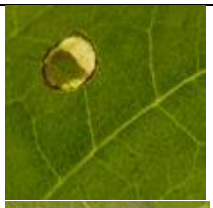
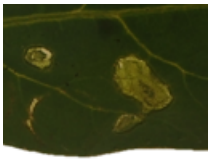
Désirée			
AVR2	PITG_10640	PITG_04099	RD2

R2				
Rpi-mcq1				
R3a				

	Bintje			
	AVR2	**PITG_10640**	**PITG_04099**	**RD2**
R2				
Rpi-mcq1				
R3a				

Appendix 4. Supplementary pictures showing the HR responses to *Désirée-R2, Désirée-Rpi-mcq1* and wild donor species upon agro-infiltrations

	AVR2	**PITG_10640**	**PITG_04099**	**RD2**

Désirée-*R2*				
Désirée-*Rpi-mcq1*				
Mcq-717-3				
Hcb-353-8				
Hjt-349-3				
Edn-151-1				

Appendix 5. Infection efficiency and lesion growth rates on the genotypes by different *P. infestans* isolates

Genotypes	Peru 4		Peru 42		Pic 99183		IPO-0		NL07434		NL09096	
	IE (%)	LGR (mm/day)	IE (%)	LGR (mm/day)	IE (%)	LGR (mm/day)	IE (%)	LGR (mm/day)	IE (%)	LGR (mm/day)	IE (%)	LGR (mm/day)
Désirée-R2	0	0	0	0	0	0	0	0	0	0	0	0
Désirée-Rpi-mcq1	0	0	0	0	0	0	0	0	86.67	1.97	50	1.24
Mcq717-3	0	0	0	0	0	0	0	0	-	-	-	-
Hcb353-8	0	0	0	0	0	0	0	0	0	0	0	0
Double-2	0	0	100	0.61	0	0	0	0	100	1.95	90	1.43
Double-4	0	0	96.67	1.89	90	1.15	0	0	-	-	-	-
Désirée-Rpi-hcb1.1-22	66.67	0.70	96.67	1.02	0	0	0	0	96.67	2.42	6.67	0.64
Désirée-Rpi-hcb1.2-3	50	0.51	100	1.8	0	0	40	1.26	100	2.38	86.67	1.85
Désirée-Rpi-hcb1.2-56	93.33	0.74	100	1.05	0	0	46.67	0.45	86.67	3.35	80	2.13
Snk213-1	0	0	0	0	0	0	0	0	-	-	-	-
Désirée	100	0.61	86.67	1.59 c	66.72	0.94	86.67	1.54	100	3.14	90	2.31

*0 or empty spaces are free from infection, (-) means not included

Appendix 6. Lesion and sporulation of different isolates on different genotypes

Peru 4

Désirée-*R2* **Désirée-*Rpi-Mcq 1*** **Hcb-*Rpi*-1.1-22** **Hcb-*Rpi*-1.2-3** **Hcb-*Rpi*-1.2-56** **Désirée**

Peru 42

Désirée-*R2* **Désirée-*Rpi-Mcq 1*** **Hcb-*Rpi*-1.1-22** **Hcb-*Rpi*-1.2-3** **Hcb-*Rpi*-1.2-56** **Désirée**

PIC 99183

Désirée-*R2* **Désirée-*Rpi-Mcq 1*** **Hcb-*Rpi*-1.1-22** **Hcb-*Rpi*-1.2-3** **Hcb-*Rpi*-1.2-56** **Désirée**

IPO-0

Désirée-*R2* **Désirée-*Rpi-Mcq 1*** **Hcb-*Rpi*-1.1-22** **Hcb-*Rpi*-1.2-3** **Hcb-*Rpi*-1.2-56** **Désirée**

NL07434

Désirée-*R2*

Désirée-*Rpi-Mcq 1*

Hcb-*Rpi*-1.1-22

Hcb-*Rpi*-1.2-3

Hcb-*Rpi*-1.2-56

Désirée

NL09096

Désirée-*R2*

Désirée-*Rpi-Mcq 1*

Hcb-*Rpi*-1.1-22

Hcb-*Rpi*-1.2-3

Hcb-*Rpi*-1.2-56

Désirée