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Monitoring and tackling genetic selection in the potato cyst nematode *Globodera pallida*

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Abstract

Management of plant pests is probably the most serious challenge in sustainable food production and the maintenance of food security. Due to the strict regulation of or ban on major categories of pesticide, the potato cyst nematode *Globodera pallida* has been managed by a combination of crop rotation and the potato resistance locus *Grp1*, a relatively narrow range resistance gene which was introgressed into a range of commercial potato cultivars in Europe. However, in 2014, *G. pallida* populations were described that can no longer be controlled by *Grp1*. Most likely similar highly virulent populations will also emerge in all major potato growing areas in North Western Europe where production practices are very similar. Except for laborious, costly and often moderately accurate pot experiments, there is currently no rapid and reliable method to identify virulent populations. This represents a strong limitation and prevents an accurate and durable management of infestations. The PalAdapt project funded by EFSA represents the first step of a European battle plan against the emergence of virulent *G. pallida* populations and aims at improving the methods and tools for a fast identification of virulence outbreaks. Four main research questions were investigated during the project: (i) Do resistance breaking populations correspond to novel introductions into Europe? (ii) Can miniaturized in vitro tests be used to get more rapidly an accurate identification of the virulence status?, (iii) Is cyst size a life history trait useful to estimate the virulence status of a population?, (iv) Can we identify polymorphism to design molecular tools for an accurate virulence monitoring? The EFSA partnering grants initiative was an accurate way to improve the EU risk assessment capacity through a knowledge exchange among partners having complementary resources and expertise.

Key words: Miniaturized tests; virulence; population genetic differentiation; allele-specific PCR; cyst size; genome assembly.

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Summary

Due to the strict regulation of or ban on major categories of pesticide, the potato cyst nematode *Globodera pallida* has been managed by a combination of crop rotation and the potato resistance locus *Grp1*, a resistance gene which was introgressed into a range of commercial potato cultivars in Europe including Iledher, Seresta, Aveka, Innovator, Cardoso, Ivetta or Amanda. However, in 2014, *G. pallida* populations were described that can no longer be controlled by any of these resistant cultivars. The PalAdapt project has involved 8 scientists from three academic partners (INRAE, France; JKI, Germany; WU, Netherlands) belonging to the top 3 potato producing countries in Europe. In order to disseminate knowledge and provide recommendations to the potato sector, the consortium conducted knowledge dissemination actions through the organisation of a workshop, the distribution of electronic newsletters to a network of 17 French, German and Dutch stakeholders and the presentation of poster and communications during national or international nematology meetings. This EFSA external scientific report intends to achieve a complete and broader dissemination of the results obtained during the project.

Virulent and avirulent populations described in Germany and the Netherlands were subjected to different molecular tools in order to investigate their genetic relationships. Our results showed that all the virulent populations of *G. pallida* found to date in Europe seem to have adapted to potato resistance from already present populations and not to novel introductions from South America. Preliminary results also suggest that a single adaptation event occurred and resulted in the outbreaks reported in Germany and the Netherlands. This conclusion is in line with the fact that the virulence probably appeared in these regions because of the intensive use of resistant varieties and the significant exchange of material between Germany and Netherlands, such as equipment.

Regarding the methods that can be used to identify virulent populations or virulent cysts in a given population, we have considered different ones. First, we looked at miniaturized virulence tests that can be used as an alternative to laborious, time consuming and costly pot experiments. Several miniaturized assays were discussed and compared during the workshop. Miniaturized and *in vitro* tests give the same results as pot tests from a qualitative point of view but not from a quantitative point of view. Such tests can then be used to get more rapidly an accurate identification of the virulence/avirulence status. Second, we looked at the life history trait "cyst size" that can be easily monitored and was shown to be impacted by the adaptation process. Our objective was to check if the correlation observed between cyst size and virulence on lineages issued from experimental evolution extend to other populations and selection events. Each partner has used different nematode material to test the universality of this observation. INRAE used Peruvian field population already able to overcome the *Grp1* potato resistance, JKI used the Emsland type field populations and WU used field populations able to overcome the *Gpa2* potato resistance. We showed that virulent field populations found in Germany tend to have bigger cysts than their avirulent counterpart. Though some exceptions were observed, cyst size seems also to be impacted the same way in Peruvian field populations able to overcome the *Grp1* resistance. However, this was not observed among populations able to overcome the *Gpa2* resistance gene. Additional experiments are clearly needed before being able to recommend the use of cyst size monitoring as a proxy of *Grp1* nematode virulence. Finally, we also investigated whether some previously identified polymorphisms can be of interest to design molecular tools for an accurate virulence monitoring. Thanks to a new genome assembly produced by WU in 2019 that can – based on primary genome statistics – truly be labelled as a reference genome, we have improved the previously published set of candidate SNPs. This updated set of candidate SNPs has been used to develop a 31 plex which was used on a Sequenom platform to genotype a total of 365 individuals representing four field populations showing different levels of virulence and four experimental lineages (both virulent and avirulent lineages). We then looked at the correlation coefficients between allele frequencies and the percentage of females' development obtained on *Grp1* resistant cultivars. Only two SNPs showed correlation coefficients above 0,55. These first results suggest that most of the outliers SNPs identified among the virulent and avirulent experimental lineages don't vary the same way among virulent field populations. Several

explanations can be considered. It should be noted that the studied virulent populations are issued from selection on different potato cultivars which show different genetic backgrounds and most probably also different combinations of genes at the *Grp1* locus. It is therefore possible that the virulence in the investigated populations rely on different genes or that the genomic pathway that led to adaptation is different in laboratory conditions compared to field conditions.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

This contract/grant was awarded by EFSA to:

Institut National de la Recherche Agronomique - Institut de Génétique, Environnement et Protection des Plantes (INRAE - IGEPP)

Contractor/Beneficiary:

- Institut National de la Recherche Agronomique - Institut de Génétique, Environnement et Protection des Plantes (INRAE - IGEPP) established in France
- Wageningen University (WU) established in The Netherlands
- Julius Kühn Institute (JKI) established in Germany

Contract/Grant title:

Novel and improved tools for monitoring and tackling genetic selection in the potato cyst nematode *Globodera pallida* populations (PalAdapt)

Contract/Grant number:

Grant Agreement Number GP/EFSA/AFSCO/2017/01 – GA08

Globodera pallida and *G. rostochiensis*, commonly known as Potato Cyst Nematodes (PCN), are nematodes parasitizing plants in the family Solanaceae, primarily infesting major crops like potatoes and tomatoes. They are not indigenous to Europe - just like the aforementioned crops - and originate from the Andes region in South America. They live on the roots of host plants and can damage them to the extent of causing growth retardation, water stress, nutrient deficiencies, early senescence of plants and ultimately yield loss. PCN are among the most highly specialized and successful plant-parasitic nematodes. They rank 2nd in the 'Top 10' list of the plant-parasitic nematodes based on their scientific and economic importance (Jones *et al.*, 2013).

Although PCN are widespread in the EU (source EPPO 2017-07-04), crop damage due to *G. rostochiensis* has so far been mild because breeders have been able to develop potato varieties that are resistant to the relatively small number of genotypes of this species that are present in Europe. The situation is different for *G. pallida* for which a wider gene pool and virulence was introduced into Europe (Folkertsma *et al.* 1996; Hockland *et al.* 2012).

Globodera pallida and *G. rostochiensis* are regulated harmful organism in the European Union. They were listed in Annex I, Part A, Section II of Council Directive 2000/29/EC. Following the evaluation of the plant health regime, the new basic plant health law, Regulation (EU) 2016/20311 on protective measures against pests of plants, was adopted on 26 October 2016 and applied from 14 December 2019 onwards, repealing Directive 2000/29/EC. The implementing act of the new plant health law,

[1] Regulation (EU) 2016/2031 of the European Parliament of the Council of 26 October 2016 on protective measures against pests of plants. OJ L 317, 23.11.2016, p. 4–104.

Commission Implementing Regulation (EU) 2019/20722, lists both nematode species in Annex II Part B, as Union quarantine pests known to occur in the Union territory.

Due to their relevance as potato pests, the control of PCN is regulated by means of a Control Directive since 1969. The Council Directive 2007/33 /EEC, whose provisions came into force from 1 July 2010, have replaced the original Control Directive 69/465/EEC "on control of Potato Cyst Eelworm". This new Control Directive establishes the measures to be taken against European populations of PCN in order to determine their distribution, to prevent their spread and to control them.

Management of pests is probably the most serious challenge in sustainable food production and the maintenance of food security. Because PCN can persist in soil for over many years, lengthy crop rotations are required to manage population levels but are not always an economically viable option. For decades, non-discriminate agro-chemicals have been used to minimize the impact of these pests. However, due to the strict regulation of or ban on major categories of pesticide (Regulation (EC) No 1107/2009, Directive 91/414/EEC), there is currently a strong demand to replace knowledge-extensive environmentally harmful chemical control strategies by more durable approaches based on natural host resistance. For decades, the PCN species *G. pallida* has been managed by a combination of crop rotation and the potato resistance locus *Grp1*, a relatively narrow range resistance gene which was introgressed into a range of commercial potato cultivars. However, in 2014, *G. pallida* populations were described from Emsland (Germany) that can no longer be controlled by *Grp1* (Niere *et al*. 2014, Fig. 1). Since then, several *G. pallida* populations have been identified from multiple locations in the north-eastern part of The Netherlands that showed aberrant multiplication rates on *Grp1* resistant starch potato varieties. These finding have been reported to the relevant EU authorities in 2015 (Dutch report entitled "NVWA Rapport fytosanitaire signaleringen 2015").

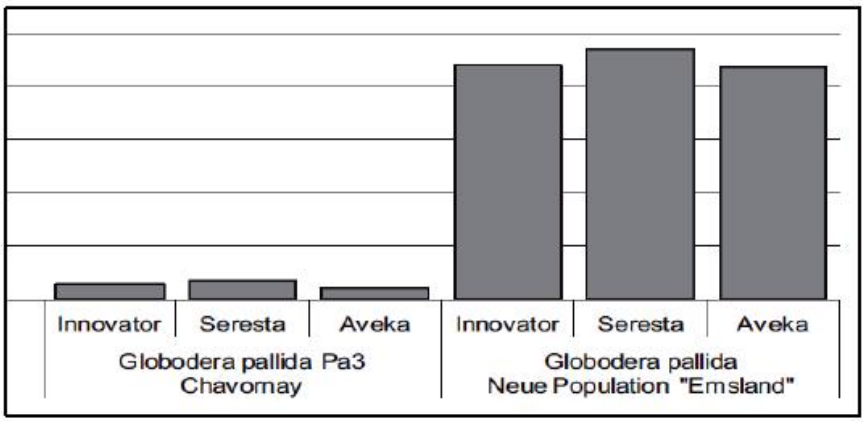

Figure 1: The effect of genetic selection by prolonged exposure to potato cultivars harboring the resistance locus *Grp1*. The Emsland population of the potato cyst nematode *G. pallida* can no longer be controlled by this major resistance gene. *Grp1* is present in potato cultivars Innovator, Seresta and Aveka (From Niere *et al*. 2014).

Except for laborious, costly and often moderately accurate pot experiments, there is currently no rapid and reliable method to identify virulent populations or virulent cysts in a given PCN population. The lack of such test represents a strong limitation to their effective management. The lack of such a reliable and transferable early warning system means that an accurate and durable management of PCNs infestations found through the only basic interception or field sampling is too difficult to contemplate. This is also crucial as the breeding of new varieties using new resistant sources, different genetic backgrounds, and/or combination of different resistant QTLs that may control these virulent populations is ongoing and will have to be evaluated regarding the levels of virulence observed in the fields in the EU.

[2] Commission Implementing Regulation (EU) 2019/2072 of 28 November 2019 establishing uniform conditions for the implementation of Regulation (EU) 2016/2031 of the European Parliament and the Council, as regards protective measures against pests of plants, and repealing Commission Regulation (EC) No 690/2008 and amending Commission Implementing Regulation (EU) 2018/2019

1.2. Project objectives

The three partners involved in this project represent the three main EU potato producers and the two member states where PCN populations with increased virulence were already reported. In 2015, 53 million tonnes of potatoes were harvested in the EU. Germany was the biggest producer, with a share of 19.5 %, ahead of France (13.4 %) and the Netherlands (12.5 %), (source Eurostat 2015). This means that, of every tonne, nearly half were grown in just these three Member States. The importance of these three Member States is even stronger when considering seed potato trade as Germany, France and Netherlands accounted for almost two thirds of intra EU exports in value terms.

The knowledge exchange conducted among the partners during the PalAdapt project represents a first step against the emergence of virulent *G. pallida* populations and aimed at improving the EU methods and tools for a fast identification of virulence outbreaks in PCN populations sampled through the different national control plans or through the potato growers monitoring plans. To achieve the potential impact of the project, our dissemination strategy was based on communications during international congress (European Society of Nematologists 2018 meeting, Organization of Nematologists of Tropical America 2019 meeting, DPG 2019 Meeting working group Nematology) and on flyer and newsletters dissemination (annex A) to various stakeholders including potato breeders and European and National Plant Protection Organisations. More, a workshop including stakeholders was organized in Wageningen in March 2019 on *in vitro* and miniaturized PCN resistance assays.

2. Data and Methodologies

2.1. Data

A total of 35 German and Dutch populations were used in this project and studied either for cyst size or molecular genotyping using microsatellite and/or AS-PCR tools. The origin and details of the use of each population during the project is indicated in the table below. Virulence status: nearly full (Vir) or severely reduced (Avir) multiplication on potato harbouring the *Grp1* locus.

Virulence status	Code	Geographic origin	Owner	Analysis conducted		
Avir	D383-1-3c	NL (2018) New standard	WU-nema	Microsat	AS-PCR	cyst size
Avir	Rook-1-2a	NL (2017) New standard	WU-nema	Microsat	AS-PCR	cyst size
Avir	D383-A5	NL (1991) Old standard	WU-nema	-	AS-PCR	-
Avir	C1-Rookmaker	NL (1991)	WU-nema	-	AS-PCR	-
Avir	Rookmaker	NL (1992) Old standard	WU-nema	-	AS-PCR	-
Avir	LD3	NL (1994)	WU-nema	-	AS-PCR	-
Avir	LD14	NL (1994)	WU-nema	-	AS-PCR	-
Avir	LD15	NL (1995)	WU-nema	-	AS-PCR	-
Avir	NOPM102G5	NL (1995)	WU-nema	-	AS-PCR	-
Avir	NOPQ53	NL (1995)	WU-nema	-	AS-PCR	-
Avir	NOPP70E5	NL (1995)	WU-nema	-	AS-PCR	-
Avir	FG22	NL (1993)	WU-nema	-	AS-PCR	-

Avir	FG25	NL (1993)	WU-nema	-	AS-PCR	-
Avir	FG28	NL (1995)	WU-nema	-	AS-PCR	-
Avir	OFLF13-S26	NL	WU-nema	-	AS-PCR	-
Avir	OFLM97w-H9	NL	WU-nema	-	AS-PCR	-
Avir	OFLL54-H13	NL (1993)	WU-nema	-	AS-PCR	-
Vir	NLBoA	NL	NVWA	Microsat	-	-
Vir	NLBoB	NL	NVWA	Microsat	-	-
Vir	NI-GPa-VIR001	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size
Vir	NI-GPa-VIR013	DE (Emsland)	JKI	-	AS-PCR	cyst size
Vir	NI-GPa-VIR012	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size
Vir	NI-GPa-VIR011	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size
Vir	NI-GPa-VIR003	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size
Vir	NI-GPa-VIR002	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size
Vir	NI-GPa-VIR004	DE (Emsland)	JKI	-	AS-PCR	cyst size
Avir	ID-174-100	DE	JKI	-	AS-PCR	-
Avir	ID-172	DE	JKI	-	AS-PCR	-
Avir	ID-321	DE	JKI	-	AS-PCR	-
Avir	ID-175	DE	JKI	-	AS-PCR	-
Vir	NL42	NL	NVWA	Microsat	AS-PCR	-
Vir	NL63	NL	NVWA	Microsat	AS-PCR	-
Avir	NI-GPa-AVI001	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size
Avir	NI-GPa-AVI003	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size
Avir	NI-GPa-AVI002	DE (Emsland)	JKI	Microsat	AS-PCR	cyst size

Several populations representing the Emsland region were studied. Emsland is a district in Lower Saxony, Germany, that is bounded on its West side by the provinces of Drenthe and Groningen, Netherlands.

2.2. Methodologies

2.2.1. AS PCR tool (INRAE methodology)

This AS-PCR tool is based on 3 single-nucleotide polymorphisms (SNPs) - SNP13441, 36915 and 5649 - allowing the distinction of the gene pool introduced into Europe (Peruvian clade I) from the other South American gene pools (Peruvian clades II to V or Chilean gene pools) (Grenier, unpublished data). The principle of AS-PCR, inspired by real-time PCR, is based on the use of two sense primers each targeting an allele (presence of the SNP of interest at the 3' end of the primer) and one antisense primer common to both alleles. Hence, two pairs of primers were designed for each of the 3 SNPs of interest. Each of the primers pair has to be used in separate reactions.

The results obtained by the AS-PCR are interpreted according to the principles of the real-time quantitative PCR which is based on the possibility to monitor amplification during the PCR process

using SYBR Green I fluorescence that has the ability to bind to nucleic acids. The fluorescence collected at each cycle of the PCR represents the amount of the amplified product at this time. Depending on the frequency of the SNP in the investigated population, the more the sample is concentrated in original target molecules, the fewer cycles it will take to reach a point for which the fluorescent signal is significantly greater than the background. This point is defined as the C_t (number of PCR cycles (C) at which a predefined threshold (t) is surpassed) and appears at the beginning of the exponential phase.

For each SNP a delta C_t (ie [C_t of allele 1] – [C_t of allele 2]) is calculated. The positive or negative value of this delta C_t allows the classification of the investigated *G. pallida* populations in one of the groups below:

a. The investigated population belongs to the Peruvian clade I gene pool (similar to the gene pool present in Europe)

b. The investigated population belongs to the Peruvian clade II to V gene pool (gene pool non-introduced into Europe)

c. The investigated population belongs to the Chilean gene pool (gene pool non-introduced into Europe).

2.2.2. Microsatellite genotyping tool (INRAE methodology)

To genotype *G. pallida* populations, we used a set of 13 microsatellite markers: Gp106, Gp108, Gp109, Gp111, Gp112, Gp116, Gp117, Gp118, Gp122, Gp126, Gp135, Gp145 and Gr67.

Locus ID	Repeat motif	Forward primer	Reverse primer
Gp106	ATTTT	TCTGTTCAGCGCACTTATGG	ATTTGATCGTTCCCTCGTTG
Gp108	AATC	TAACGGCTATCAGCCCAATC	TCGGCCAAAACGTAAAACTC
Gp109	ACGG	TCTCGCAGAAGGGAAAAGAA	TAAAAGACGGAAGAACGGGA
Gp111	TCGG	TCCATTTGTTTTGGGGACAT	CCGTGTCCGATAAATTCCTG
Gp112	AATG	GTTTTAAGCAGACAAGGCCG	ATCTCATAGCAATTTGCCCG
Gp116	CGTC	ATTCATTCGCAATGTTTCCC	TGGAAATGTGAGAAAGGGCT
Gp117	GCCC	GTCTATTGGCGGCACGTATT	TTCCAAATCCGCCATAATTG
Gp118	TCCG	ACCGATGAAGAACATCGTCC	TCGTTCCGTCTTCGTAATCC
Gp122	CATT	AGAGGGTGCCTTTGCTTCTT	ATTGAGTGCCAATAATCCGC
Gp126	GATT	GTTATTGTGGCGGATGGAAT	GTACTGTATGATGCCGGGCT
Gp135	GA	GCGAAATGAACGGTCGTAGT	ATTACATTGCCCAAATCGGA
Gp145	CGTC	TCACAAAACATTGGAGCTGAA	TTAAATGAGAAACGGGGGAAT
Gr67	GT	ACCTGAACGTCGTCATTTCC	TTTTCTTACCCGAATGGCAC

These markers were multiplexed in two panels. The panel 1 include Gp106 (the forward primer being labelled in blue, i.e. FAM), Gp109 (labelled in yellow, i.e. NED), Gp111 (FAM), Gp118 (labelled in red, i.e. PET), Gp135 (labelled in green, i.e. VIC) and Gp145 (VIC). The panel 2 include Gp108 (FAM), Gp112 (NED), Gp116 (NED), Gp117 (PET), Gp122 (FAM), Gp126 (PET) and Gr67 (VIC). For each panel, PCR was performed using a 96-well reaction module in a 10 µL volume containing Type-it Microsatellite PCR kit (QIAGEN), the primer mix and the template DNA. PCR products were then diluted in sterile water and mixed with GeneScan 500 LIZ Size Standard and formamide.

All the PCR products were run on an ABI Prism® 3130xl sequencer (Applied Biosystems) at INRAE facilities. Allele sizes were determined by the automatic calling and binning module of GeneMapper v4.1 with manual examination of irregular results. To minimize the rate of genotyping errors, a second round of PCR and electrophoresis was performed for 10% of the global number of individuals.

2.2.3. Miniaturized and *in vitro* virulence testing

In vitro nematode resistance tests (WU methodology)

For the *in vitro* nematode resistance tests, which were demonstrated during the workshop and used to determine the cyst size results and infection levels, the avirulent *G. pallida* population D383 and the virulent population Rookmaker were used for infection of transgenic potato lines carrying the *Gpa2* gene. Non-transformed lines were used as susceptible control. Stem cuttings of *in vitro* potato plants were grown on 1% B5 agar plates (one per plate) under normal light conditions, and after 3 weeks, roots were infected with approximately 150 surface-sterilized second stage juveniles (J2) per plate and further incubated in the dark at 18°C. Surface-sterilized pre-parasitic J2's were obtained from dry cysts and hatching was promoted by filter-sterile potato root diffusate that was collected by allowing potato roots to grow in tap water for 4 weeks in the dark. After 3 to 5 days, J2's were collected on a 5-µm-pore-size sieve and surface-sterilized using the following disinfectants: 0.5% (wt/vol) streptomycin sulphate-penicillin G (20 min), 0.1% (wt/vol) ampicillin-gentamycin (20 min), sterile tap water (5 min), and 0.1% (vol/vol) chlorhexidine-digluconate (3 min). After washing in sterile tap water, J2's were suspended and counted to determine the inoculation density. For each treatment, at least 5 repeats were used. After 28 days, nematode development was monitored by microscopic inspection and pictures were made for determining the size of adult females. In addition, the total number of females present on the roots was counted to test for difference in resistance and susceptibility.

Potato plants Tissue Culture (TC) for phenotyping resistance in potato genotypes (JKI methodology)

In vitro propagated TC potato plants and potato tubers were used in this study. Pots were filled with Loess soil and the plantlets carefully transplanted to cover the entire root system. To maintain high relative humidity suitable for plant establishment, TC plants were loosely covered with a clear polythene cover for 7 days after which the cover was gradually removed. When planting tubers or eye-plugs, pots were half filled with soil and the planting material placed at the middle of the pot. Pots were then filled with soil and placed on the glasshouse bench. Fourteen days after planting, each pot was inoculated with *G. pallida* eggs and J2's. During the inoculation, two holes, 3 cm deep, were made in the soil using a plastic rod and the nematode suspension dispensed in the holes to achieve a Pi of 5 eggs and J2's/ml soil. Plants were watered as needed and maintained in the glasshouse for a period of 12 weeks after inoculation giving a total of 14 weeks from planting to the termination of the experiments. The figure 2 shows the setup using TC plants and how plants develop in the plastic folded boxes.

A


B


C


D

Figure 2: A= tissue culture plants received from breeders; B = tissue culture plants in small plastic folded boxes (4x4cm) 2 weeks after transplanting into Loess soil; C = different genotypes of potato 10 weeks after transplanting; D = white females developing on the root.

With this method developed by Mwangi *et al.* (2019), virulent populations can be characterized and their interaction with the host plant investigated. In addition, this method allows for a high throughput phenotyping of potato genotypes, using TC plantlets.

Petri dish test for phenotyping resistance in potato genotypes (INRAE methodology)

This Petri dish test allows, in a simple and quick way, to evaluate the virulence level of a given PCN population and/or the resistance level of a potato cultivar. Germinated pieces of potato were deposited on a 20 g/l agar in Petri dishes (prepared 5 days before). Each root was inoculated with ten newly hatched J2's in the three days after (roots must not exceed 2 cm long). Petri dishes were then stored at 20°C in the dark to allow adult development. After 15 days, roots were cut with a scalpel and dissected in water under a binocular magnifier. Avirulent populations are expected to give only a few adults and or a majority of males on resistant cultivars compared to a susceptible one which will conversely develop a majority of females (around 80%). On the opposite, virulent populations are expected to give more females than avirulent populations on resistant cultivars. The difference in terms of number of females obtained between the resistant cultivar and the susceptible control may be less significant or equal when the virulence level of the population is very high.

2.2.4. Estimating cyst size as a life history trait of interest in adapted populations of *G. pallida*

Regarding the study conducted on German populations: the size (diameter) of the cysts of *G. pallida* 'Oberlangen' (NI-GPa-VIR001) and other populations were measured using a Nikon® SMZ18 Stereo Zoom Microscope. Three measurements were taken per cyst for 40 randomly picked cysts per population. The size of the cysts of six other virulent populations and three avirulent populations was determined as described above. These populations had been obtained at different times during field surveys in the Emsland region of Lower Saxony and their virulence determined. However, unlike 'Oberlangen', they were not reproduced in the glasshouse prior to the study.

Regarding the study conducted on Peruvian populations: All the cysts used originated from the same multiplication cycle on the susceptible cultivar Désirée. To achieve that ten cysts (on average 1500 individuals) were placed in a 13 cm pot three-quarter filled with a soil mixture (1/3 sand and 2/3 natural field soil). One tuber of Désirée was then planted and covered with the same soil mixture. Potatoes were grown until they die (around 120 days) under controlled conditions (20 +/- 4°C). Newly formed cysts were extracted from the soil using a Kort elutriator and stored at 4°C for a minimum of two months. Each population was multiplied in five independent replicates. Average cyst size of each population was determined on 300 cysts randomly chosen from the pool corresponding to the five multiplication replicates. The surface of each cyst was measured using a magnifying stereomicroscope coupled with an image analysis software (Microvision Intruments, Histolab V8.10, Evry, France). For each population, cysts larger than (named "lk" for large cysts) or smaller than (named "sk" for small cysts) the average plus or minus the standard error, respectively, were kept separately. The virulence level was subsequently evaluated on 40 randomly chosen cysts taken either from the small or the

large cyst pools and using the Petri dish test (see description in 2.2.3). At least, 10 independent roots were inoculated per tested condition (ie small or large cysts) and were stored at 20°C in the dark for 15 days to allow adult development.

2.2.5. New assembly of the *G. pallida* genome: an improved resource for virulence molecular markers (WU ressource)

As part of an ongoing project at WU the genome of a Dutch population of *G. pallida* was sequenced. The population in question, D383, is not able to overcome known resistances - as *Grp1* (discovered in potato AM78-3778) and *Gpa2* (discovered in potato SH82-93488) - against potato cyst nematodes. The genome was sequenced using PacBio chemistry. During assembly, special care was taken to account for the increased heterozygosity that is inevitably present when sequencing a population. An additional round of HiSeq sequencing was performed to improve the final polishing phase of the assembly and to perform variant calling on the final assembly of the genome.

After finishing the assembly, the annotation process was started. First repetitive DNA sequences were identified. After checking these sequences did not show a resemblance to known genes or non-coding RNA, these repeats were masked. Next, non-coding RNA was identified, annotated and masked. Subsequently a structural gene annotation was performed using two different annotation programs in parallel: Maker 2.31.10 and Braker 2.1.4. As part of this process, all publicly available RNAseq data for *G. pallida* was mapped against the genome assembly. The annotation programs used a combination of comparison with known genes from closely related species, mapped RNAseq data and *de novo* gene prediction to come to a gene annotation. The resulting gene annotations were compared with each other and overlapping loci were identified. A combined structural gene annotation was created by selecting the annotation that best matched the mapped RNAseq data for each locus.

The final genome assembly was shared with the project partners. A genome browser was set up using the Apollo 2.4.0. This genome browser visualizes the genome and the structural annotation. It also displays the mapped RNAseq data and variant call information.

3. Assessment/Results

- Do resistance breaking populations correspond to novel introductions into Europe? Do the German and Dutch variants correspond to single or different adaptation events?

  Our objective was to identify the origin of resistance breaking PCN populations found in Germany and the Netherlands. In that aim, a first knowledge transfer action corresponding to the sharing with the partners of an unpublished Allele-Specific (AS-PCR) molecular tool developed by INRAE was carried out successfully. Using this tool, JKI and WU were able to show that the virulent populations found in Germany and in the Netherlands since 2014 belong to the European gene pool. Therefore, all the virulent populations of *G. pallida* found to date in Europe seem to have adapted to potato resistance from already present populations and not to novel introductions from South America.

	Virulence status	Code	Geographic origin	SNP13441 Delta C_t (T-A)	SNP36915 Delta C_t (G-A)	SNP5649 Delta C_t (G-T)
WU Analysis	Avir	D383-1-3c	NL (2018) New standard	11,1	-9,2	-3,5
	Avir	Rook-1-2a	NL (2017) New standard	9,5	-9	-3,5
	Avir	D383-A5	NL (1991) Old standard	14,9	-13,6	-15,4
	Avir	C1-Rookm	NL (1991)	9,6	-11,3	-4,1

	Avir	Rookmaker	NL (1992) Old standard	10,5	-10	-3,7
	Avir	LD3	NL (1994)	11,2	-12,1	-5,9
	Avir	LD14	NL (1994)	10,2	-10,2	-4,6
	Avir	LD15	NL (1995)	8,7	-9,6	-3,9
	Avir	NOPM102G5	NL (1995)	12,1	-15,4	0
	Avir	NOPQ53	NL (1995)	11,6	-18,5	-5,6
	Avir	NOPP70E5	NL (1995)	14,7	-11,5	-6,4
	Avir	FG22	NL (1993)	12,7	-12,5	-6,3
	Avir	FG25	NL (1993)	8,2	-13,9	-5,2
	Avir	FG28	NL (1995)	11,4	-8,8	-4,1
	Avir	OFLF13-S26	NL	13,5	-10	-4,9
	Avir	OFLM97w-H9	NL	10,4	-10,1	-7,2
	Avir	OFLL54-H13	NL (1993)	13,8	-14	-5,3
	Vir	NLBoA	NL			
	Vir	NLBoB	NL			
	European genepool std	Chavornay	CH	14	-16,8	-5,9
	Peruvian genepool std	Chocon	PE	-3,6	-8	-3,4
	Chilean genepool std	Terre de Feu	CL	11,2	13,5	7,5
JKI analysis	Vir	NI-GPa-VIR001	DE (Emsland)	5,1	-15,8	-10,8
	Vir	NI-GPa-VIR013	DE (Emsland)	14,1	-15,8	-10,7
	Vir	NI-GPa-VIR012	DE (Emsland)	15,6	-16,2	-11,9
	Vir	NI-GPa-VIR011	DE (Emsland)	13,9	-15,9	-9,5
	Vir	NI-GPa-VIR003	DE (Emsland)	14,7	-16,3	-9,9
	Vir	NI-GPa-VIR002	DE (Emsland)	15,4	-16	-10,5
	Vir	NI-GPa-VIR004	DE (Emsland)	14,4	-16,6	-8,6
	Avir	ID-174-100	DE	14,5	-14,3	-10
	Avir	ID-172	DE	16,1	-16,1	-11,8
	Avir	ID-321	DE	15,3	-10,9	-10,2
	Avir	ID-175	DE	16,8	-14,8	-11,7
	Vir	NL42	NL	13,1	-15,3	-10,2
	Vir	NL63	NL	14,7	-12,8	-12,2
	Avir	NI-GPa-AVI001	DE (Emsland)	14,9	-11,7	-11
	Avir	NI-GPa-AVI003	DE (Emsland)	17	-13,6	-9,9
	Avir	NI-GPa-AVI002	DE (Emsland)	10,3	-14,5	-10,8

	European genepool std	Chavornay	CH	15	-15,4	-11,4
	Peruvian genepool std	ID-188	PE	-18,7	-17,2	-6,9

Table 1: AS-PCR results obtained by the project partners on field populations collected in the Netherlands and in Germany including some populations able to overcome the *Grp1* resistance. Control samples are highlighted in yellow. All PCR were conducted in replicates (data not shown).

A second knowledge transfer action took also place between the partners. A genotyping assay using a set of 13 neutral microsatellite markers that allows the distinction between geographically different populations that experienced two independent selection process in INRAE tests was shared and even improved thanks to the procedures used by the partners for single larvae DNA extractions and the reaction cost was even lowered thanks to the experience of the partners with other fluorophores. The genotyping assay based on 13 microsatellite markers was used by the JKI and WU to genotype virulent and avirulent *G. pallida* populations from the same geographic area. The STRUCTURE analysis conducted on the genotyping data revealed two genetic clusters (Fig. 3), but each population included individuals assigned to each cluster. Individuals of the population NL42 were all assigned to only one genetic cluster and this population was the less diverse one (H_{nb} = 0.276; Table 2) showing that cysts at the origin of this population were coming from one more genetically diverse population (one of the other sampled population, or an unsampled population) harbouring individuals assigned to both clusters. This preliminary result suggests that a single adaptation event occurred and resulted in the outbreaks reported in Germany and the Netherlands. This conclusion is in line with the fact that the virulence probably appeared in these regions because of the intensive use of resistant varieties. Starch potatoes are grown every two years in the Emsland region and there was a significant exchange of material between DE and NL, such as equipment, in this region. However, as few Dutch virulent populations and no Dutch avirulent population were used in this first investigation this first result will need to be confirmed. The WU partner is currently genotyping other Dutch populations to strengthen these first conclusions.

Population	**Location**	**Virulence status**	***FIS***	**H_{nb}**	**Mean allele nb / locus**
NI-GPa-VIR012	Germany	Vir	0,05	0,46	3,00
NI-GPa-AVI002	Germany	Avir	0,28	0,46	3,31
NI-GPa-VIR011	Germany	Vir	0,18	0,47	3,38
NI-GPa-AVI003	Germany	Avir	0,19	0,50	3,38
NI-GPa-VIR002	Germany	Vir	0,19	0,49	3,08
NL63	Netherlands	Vir	0,14	0,47	3,31
NL42	Netherlands	Vir	0,20	0,28	2,15
NI-GPa-VIR001	Germany	Vir	0,25	0,46	3,23
NI-GPa-AVI001	Germany	Avir	0,04	0,46	3,38
NI-GPa-VIR003	Germany	Vir	0,19	0,47	3,15

Table 2: Genetic diversity statistics obtained for Dutch and German virulent and avirulent populations. F_{IS} shows the departure from Hardy–Weinberg equilibrium, H_{nb} corresponds to the unbiased gene diversity.

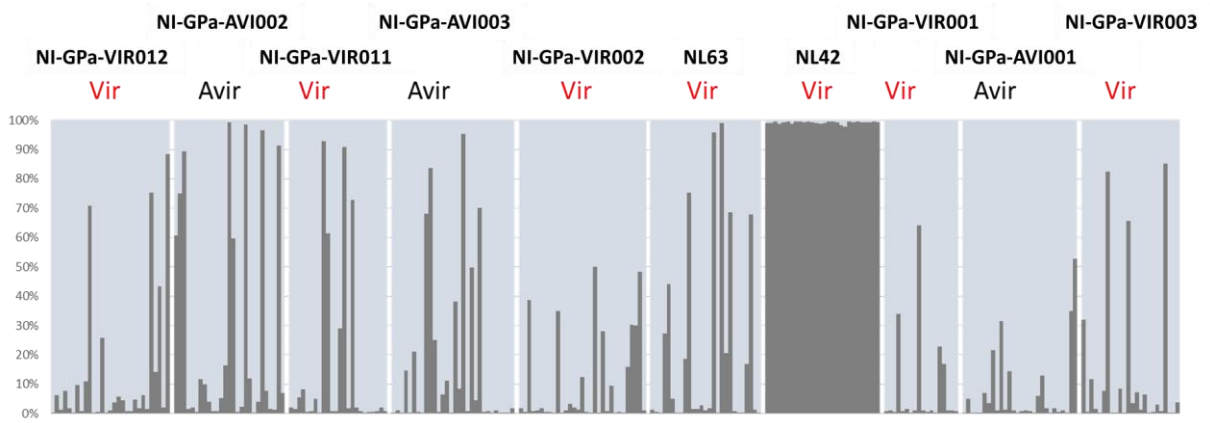

Figure 3: STRUCTURE graph showing the genetic structure obtained using Dutch and German virulent and avirulent populations. The y-axis shows the assignation rate to the genetic clusters of each individual displayed on the x-axis (35 individuals were genotyped per population).

- Can miniaturized *in vitro* tests be used to get more rapidly an accurate identification of the virulence status?

  A workshop entitled "*in vitro* and miniaturized cyst nematode resistance assays on potato: current use and future prospects" was held from 18 to 20th March 2019 in Wageningen. It was attended by 19 participants, including PalAdapt stakeholders affiliated to either potato breeding companies, NPPO, national safety organizations or reference laboratories for nematode resistance testing in potato.

  In vitro and miniaturized nematode infection tests are significant shorter than greenhouse test and allows more detailed observation of the nematode-host interaction. Root obtained from *in vitro* stem cuttings can be infected with nematodes already after three weeks, whereas it takes about 2 months to grow a potato plant (from *in vitro* stocks) in the greenhouse before inoculation. The development of the nematodes and the plant response can be monitored during the infection process using a binocular which is impossible for greenhouse grown plants in a non-destructive manner. Finally, adult females can be counted to determine the virulence level of the nematode population used and/or the resistance level of the host plant in an early stage (28 days post inoculation) compared to cysts harvested from greenhouse grown plants after completion of their life cycle (3 month after inoculation). Both methodologies are often used in combination with similar results, for example to test plant materials first in the lab before validation of a specific subset of genotypes in the greenhouse. Examples are described in a number of publications (Goverse *et al.* 2000; Slootweg *et al.* 2018).

  Main conclusions were: (i) miniaturized tests can be used to get more rapidly an accurate identification of the virulence status, (ii) miniaturized and *in vitro* tests give the same results as pot tests from a qualitative point of view (*i.e.* the population is or is not virulent) but not from a quantitative point of view (*i.e.* ranking and levels of virulence can differ), (iii) overall, the different methodologies presented already allow the addressing of a large panel of situations and questions.

- Is the life history trait "cyst size" useful to estimate the virulence status of a population?

  Our objective was here to check if the correlation observed between cyst size and virulence on lineages issued from experimental evolution (Fournet *et al.*, 2016) extend to other populations / selection events. Each partner has used different nematode material to test the universality of this observation. INRAE used Peruvian field population already able to

overcome the *Grp1* potato resistance, JKI used the Emsland type field populations and WU used field populations able to overcome the *Gpa2* potato resistance gene. The results obtained show that virulent field populations found in Germany tend to have bigger cysts than their avirulent counterpart (Fig. 4). Additional experiments are planned for 2020 to further investigate this correlation and the interest of cyst size monitoring as a proxy of *Grp1* nematode virulence.

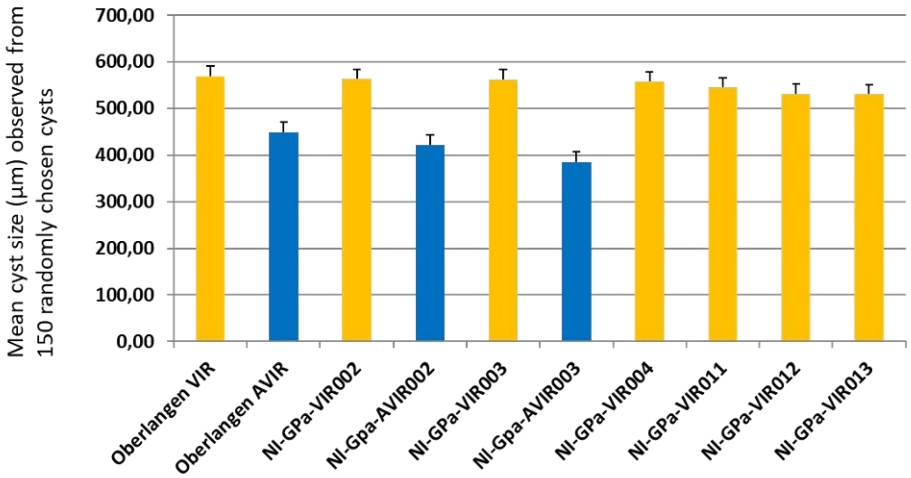

Figure 4: Cyst size of virulent and avirulent German field populations.

Though some exceptions were observed, cyst size seems also to be impacted the same way in Peruvian field populations able to overcome the *Grp1* resistance and representing several independent process of resistance adaptations (Fig. 5). Mean cyst size among Peruvian populations can vary greatly (22,3 x 10^4 to 27,5 x 10^4 µm^2) and we observed that the trend between *Grp1* virulence and cyst size is stronger for populations having a low mean cyst size than for populations having a high mean cyst size.

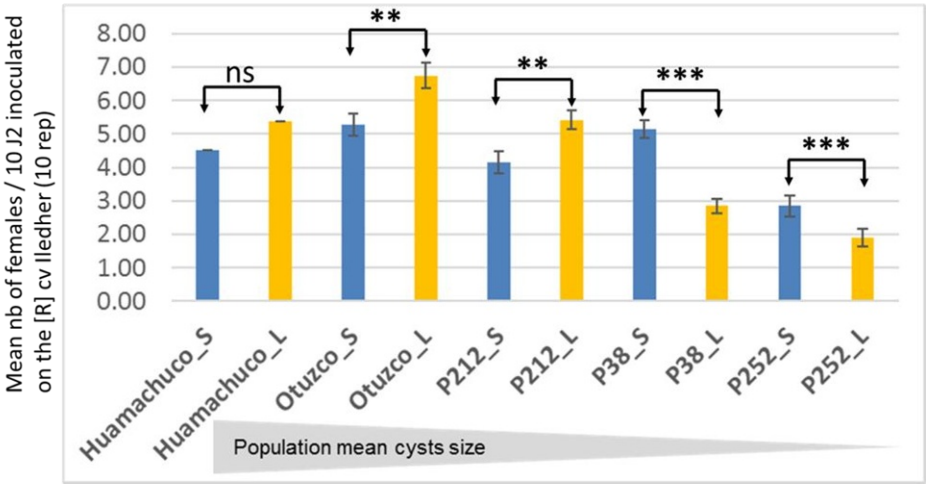

Figure 5: Virulence levels of small (S) and large (L) cysts of North Peruvian populations according to their mean population size. Asterisks indicate difference within population between virulence levels of S and L cysts (* p<0.05 ; ** p<0.01 ; ***p<0.001).

To investigate whether there is a difference in size between other type of virulent and avirulent populations, an infection assay was performed on *in vitro* grown roots of a susceptible potato genotype Line V with or without the single dominant R gene *Gpa2*. Our data (Fig. 6) indicate that there is no difference in size when comparing adult females from the virulent (Rookmaker) and avirulent (D383) populations. On *Gpa2* resistant roots, as expected D383 did not develop properly only resulting in a few underdeveloped females which explains their reduced size. Interestingly, our data show also a slight reduction in the size of adult females of Rookmaker compared to the females developing on the susceptible line (Fig. 6). Further experiments are needed to see if this is reproducible and what the underlying cause is.

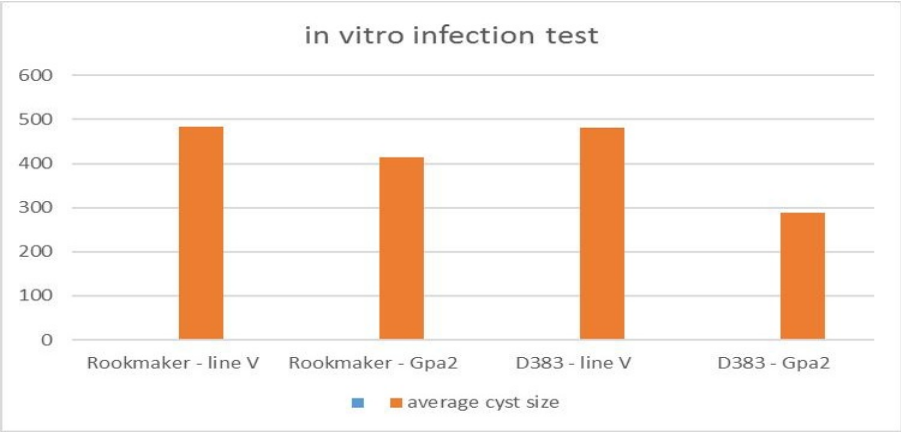

Figure 6: *In vitro* infection assay on potato roots of carrying the resistance gene (*Gpa2*) and control lines lacking this gene (Line V). Roots were infected with surface sterilized juveniles of the virulent *G. pallida* population Rookmaker or the avirulent population D383. Average size (µm) of adult females (cysts) was determined by binocular for 20-30 individuals randomly selected from five plates one month after inoculation.

- Can we identify polymorphisms to design molecular tools for an accurate virulence monitoring?

  A knowledge transfer firstly took place with the presentation and regular update regarding the assembly of the new *G. pallida* genome. By September 2019 the version called G_pallida_D383_v.0.7.d2b.fasta was considered as sufficiently improved and robust to start working with it. As demonstrated by Table 3, the new genome assembly is a considerable improvement over the earlier assembly and the published nematode genome. The assembly size has been reduced and now approaches the size estimated by flow cytometry (S. Eves-van-den-Akker, pers. com.). It consists of fewer and larger scaffolds (6,873 -> 1,610 -> 163). The heterozygosity in the data, an inevitable side effect of having to sequence a population, was successfully dealt with in the assembly. This is demonstrated by low amount of duplicated fragments in the BUSCO score. The BUSCO score is a measure of completeness of the genome, as it searches the genome for single copy genes that are highly conserved and present in all eukaryotes. WU granted access to the partners to this version including via the user-friendly Apollo genome browser. This includes structural annotation, mapped RNAseq data and variant calling information. Having a high-quality genome available means that other populations can be re-sequenced using the much cheaper HiSeq chemistry. Mapping this data against the new reference genome will allow for the characterization of genetic differences between populations differing in virulence.

Assembly	Size (Mb)	scaffolds	N50 (Mb)	Busco single	dupl.	frag.	missing
Cotton *et al.* (2014)	123	6873	0.12	63.7	5.0	12.5	18.8
WU old (2019)	153	1610	0.28	63.0	21.8	7.3	7.9
WU new (2020)	113	163	2.86	78.9	0.7	10.6	9.8

Table 3: Genome statistics and BUSCO scores of the published genome by Cotton *et al.* (2014) and the old and new assembly of the WU genome

As indicated during the project kick off meeting, the objective was to identify molecular markers for an accurate and delegable virulence monitoring. During the PalAdapt project we have used the assembly of the new *G. pallida* genome to improve the actual set of candidate SNPs identified previously at INRAE (Eoche-Bosy *et al.*, 2017). This updated set of candidate SNPs has been used to develop a Sequenom 31-plex composed of 10 candidate SNPs supported by both the initial and new version of the genome assembly, 13 candidate SNPs supported by only the new version of the genome assembly and 8 candidate SNPs supported by only the initial version of the genome assembly. First results came in just at the end of the project and analysis is still underway. A total of 365 individuals representing four field populations showing different levels of virulence and four experimental lineages (2 virulent and 2 avirulent lineages used to identify the candidate SNPs) were used in this first Sequenom genotyping. Several unexpected results were observed. First a high rate of amplification failure was observed with 14 SNPs showing no or less than 5% amplification success among the 365 individuals tested. Second, among the 17 remaining SNPs, 4 appear surprisingly monomorphic in all the tested populations including the experimental lineages from where they were initially identified. In order to investigate the impact of the number of genotyped larvae on the population allele frequency estimate, we have genotyped 90 individuals in a given population showing a medium virulence on *Grp1* resistant cultivars. The results obtained (Fig. 7) showed that reducing the number of genotyped larvae to 40 individuals per population will still allow a good assessment of the population allele frequency (< 4% error).

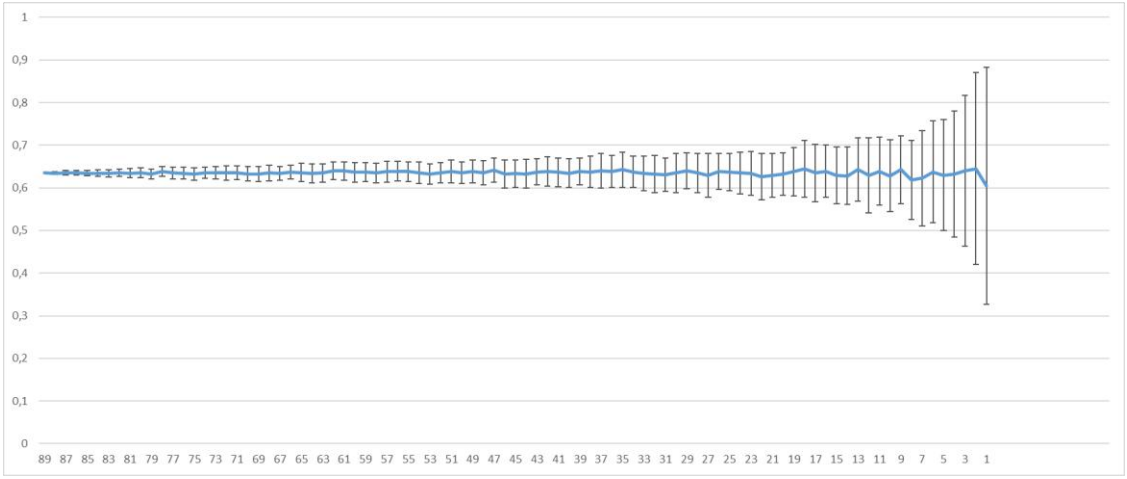

Figure 7: Mean and standard error of the allelic frequency (y-axis) calculated using 100 random replicates according to the number of individuals used (x-axis). The SNP DEB338_98803 was used here as an example.

Using the 13 remaining polymorphic SNPs we have looked at the correlation coefficients between allele frequencies and the % of females' development obtained on *Grp1* resistant cultivars. The best correlation scores observed (0,55 and 0,61) were observed for two SNPs identified in the initial version of the genome assembly (SNP340_56189 and SNP349_8349) but found absent in the new genome assembly. Despite half of the populations investigated in this first Sequenom analysis correspond to the experimental lineages used to identify the

SNPs, 11 SNPs show coefficient correlations below 0,33 suggesting that the correlation with the phenotypic data drastically decreases when the 4 field populations are included in the analysis. These first results suggest that most of the outliers SNPs identified among the virulent and avirulent experimental lineages don't vary the same way among virulent field populations. Several explanations can be considered. It should be noted that the studied virulent populations are issued from selection on different potato cultivars which show different genetic backgrounds and most probably also different combinations of genes at the *Grp1* locus. It is therefore possible that the virulence in the investigated populations rely on different genes or that the genomic pathway that led to adaptation is different in laboratory conditions compared to field conditions.

4. Conclusions

1. All the virulent populations of *G. pallida* found to date in Europe seem to have adapted to potato resistance from already present populations and not to novel introductions from South America.
2. Preliminary results suggest that a single adaptation event occurred and resulted in the outbreaks reported in Germany and the Netherlands.
3. Miniaturized virulence tests can be used to get more rapidly an accurate identification of the virulence status
4. Miniaturized and *in vitro* tests give the same results as pot tests from a qualitative point of view but not from a quantitative point of view
5. There is a clear need to generate an updated list of *G. pallida* reference populations as test panel for our future assays
6. A new *G. pallida* genome was made available that can – based on primary genome statistics – truly be labelled as a reference genome
7. Most of the outliers SNPs identified among the virulent and avirulent experimental lineages don't vary the same way among virulent field populations.

5. Recommendations

Potato cyst nematodes are present in all potato producing regions in Europe, and there is no reason to consider that the presence of virulent populations of PCN will remain limited to the actual countries represented in PalAdapt. Therefore, we recommend that the collaboration established through the current EFSA grant should be expanded and broadened.

Expansion of the collaboration is required for the elucidation of the molecular basis of virulence towards *Grp1* in *G. pallida*. A Europe-wide characterisation of avirulent and virulent populations by re-sequencing could very much contribute to this goal. In this perspective Nem-Emerge, a proposal led by WU and involving several partners including JKI and INRAE, was submitted to the H2020-SFS-05-2020 call "New and emerging risks to plant health".

In parallel to re-sequencing, a more affordable and easier interpretable genotyping tool could be instrumental to broaden the use of molecular tools for monitoring changes in population structure, gene pools and virulence for effective PCN control. We suggest that future reports of virulent populations in the EU will be analysed by the afore-mentioned tools. In order to build a genotype repository for European populations and monitor the occurrence of possible independent adaptation events, newly generated data should preferably be added to ones obtained in this project.

We also intend to seek support to organize again a workshop on PCN resistance tests using miniaturized systems. Now the workshop was targeting stakeholders of PalAdapt partners, but there is a clear interest in the broader community to learn more about these methods, to get training and to share expertise. In addition to a wider use, this will also allow more standardization of tests among labs and thus comparison of data.

Finally, this EFSA project made clear that there is a need for an updated list of *G. pallida* reference populations as test panel for our future assays. To improve the confidence of the resistance scoring across Europe, instructions should be provided regarding the rearing and control of the reference populations. As a consortium we will investigate in 2020 whether genetic drift has resulted in a diversification of the widely used standard *G. pallida* population Chavornay among the partner laboratories. Furthermore, we will plan to investigate whether the German 'Oberlangen' (NI-GPa-VIR001) population maintained by JKI can be recommended as a new standard *G. pallida* population.

We should be able to provide new recommendations on *G. pallida* reference populations by the end of this year.

References

Cotton JA, Lilley CJ, Jones LM *et al*. The genome and life-stage specific transcriptomes of *Globodera pallida* elucidate key aspects of plant parasitism by a cyst nematode. Genome Biol. 2014; 15: R43.

Eoche-Bosy D, Gauthier J, Juhel AS, Esquibet M, Fournet S, Grenier E, Montarry J. Experimentally evolved populations of the potato cyst nematode *Globodera pallida* allow the targeting of genomic footprints of selection due to host adaptation. Plant Pathol. 2017; 66(6): 1022-30.

Eoche-Bosy D, Gautier M, Esquibet M, Legeai F, Bretaudeau A, Bouchez O, Fournet S, Grenier E, Montarry J. Genome scans on experimentally evolved populations reveal candidate regions for adaptation to plant resistance in the potato cyst nematode *Globodera pallida*. Mol Ecol. 2017; 26(18): 4700-4711.

Folkertsma RT, Rouppe van der Voort JN, deGroot KE, van Zandvoort PM, Schots A, Gommers FJ, Helder J, Bakker J. Gene pool similarities of potato cyst nematode populations assessed by AFLP analysis. Mol Plant-Microbe Interact. 1996; 9(1): 47-54.

Fournet S, Eoche-Bosy D, Renault L, Hamelin FM, Montarry J. Adaptation to resistant hosts increases fitness on susceptible hosts in the plant parasitic nematode *Globodera pallida*. Ecol Evol. 2016; 6(8): 2559-68.

Fournet S, Kerlan MC, Renault L, Dantec JP, Rouaux C, Montarry J. Selection of nematodes by resistant plants has implications for local adaptation and cross-virulence. Plant Pathol. 2013; 62(1): 184-93.

Goverse A, Overmars H, Engelbertink J, Schots A, Bakker J, Helder J. Both induction and morphogenesis of cyst nematode feeding cells are mediated by auxin. Mol. Plant–Microbe Interact. 2000; 13: 1121–1129.

Hockland S, Niere B, Grenier E, Blok V, Phillips M, den Nijs L, Anthoine G, Pickup J, Viaene N. An evaluation of the implications of virulence in non-European populations of *Globodera pallida* and *G. rostochiensis* for potato cultivation in Europe. Nematology. 2012; 14(1): 1-13.

Jones JT, Haegeman A, Danchin EGJ, Gaur HS, Helder J, Jones MGK, Kikuchi T, Manzanilla-Lopez R, Palomares-Rius JE, Wesemael WML, Perry R. Top 10 plant-parasitic nematodes in molecular plant pathology. Mol Plant Pathol. 2013; 14(9): 946-61.

Mwangi JM, Niere B, Daub M, Finckh MR, Kiewnick S. Reproduction of *Globodera pallida* on tissue culture-derived potato plants and their potential use in resistance screening process. Nematology 2019; 21: 613-623.

Niere B, Krüssel S, Osmers K. Auftreten einer außergewöhnlich virulenten Population der Kartoffelzystennematoden. Journal für Kulturpflanzen. 2014; 66(12): 426-427.

Plantard O, Picard D, Valette S, Scurrah M, Grenier E, Mugniéry D. Origin and genetic diversity of Western European populations of the potato cyst nematode (*Globodera pallida*) inferred from mitochondrial sequences and microsatellite loci. Mol Ecol. 2008; 17(9): 2208-2218.

Sacco MA, Koropacka K, Grenier E, Jaubert MJ, Blanchard A, Goverse A, Smant G, Moffett P. The Cyst Nematode SPRYSEC Protein RBP-1 Elicits *Gpa2*- and *RanGAP2*-Dependent Plant Cell Death. PLoS Path. 2009; 5(8): e1000564.

Slootweg EJ, Spiridon LN, Martin EC, Tameling WIL et al. Distinct Roles of Non-Overlapping Surface Regions of the Coiled-Coil Domain in the Potato Immune Receptor Rx1. Plant Physiol. 2018; 178(3): 1310-1331