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Quantitative data on the impact of resistance genes on the population dynamics of P. infestans

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Contents

Abstract

The purpose of AMIGA DL 8.4 is to deliver quantitative data on the selective impact of host resistance genes on the population dynamics of *P. infestans*. Selection pressure causes undesirable shifts in the pathogen population, e.g. towards virulence for specific (combinations of) R-gene(s). Presence of selection pressure indicates the critical need for continuous pathogen population monitoring and a dynamic control strategy to counteract the negative effects of selection pressure.

Significant differences between the local pathogen populations in Carlow (Ireland) and Valthermond (the Netherlands) were found: the Carlow population is relatively stable over years and primarily made up of a few well known clonal lines whereas the Valthermond population is highly dynamic over years, contains greater genetic variation and is primarily made up of genetically distinct genotypes plus contributions from clonal line EU_13_A2. The difference in genetic variation contained in both populations is very likely due to the effect of an active sexual cycle in Valthermond.

As expected, resistant cultivar Sarpo Mira and resistant, Desiree derived, GM potato clone A15-031 both exerted significant selection pressure on both local pathogen populations. In Carlow, Sarpo Mira was infected by a limited number of specific sub-variants of clonal line EU_13_A2. In Valthermond, Sarpo Mira was also infected by sub-variants of EU_13_A2 plus a range of genetically distinct isolates. Vnt1 containing potato clones were only infected in Valthermond by a range of genetically distinct pathogen genotypes outside the well-known clonal lines. Populations harbouring high levels of genetic variation are thus more likely to overcome R genes. This also illustrates the high potential for rapid change towards a virulent pathogen population once the *Rpi-Vnt1* is used on larger acreages.

Investigation of specific selection pressure exerted by GM crops versus non-GM crops remained inconclusive. Insufficient samples were found on the conventional Vnt1 clone, factors “year” and “origin” were intertwined and the pathogen population in Valthermond demonstrated a large background variability between years.

It is concluded that cultivation of (GM) resistant potato exerts significant selection pressure on the local *P. infestans* population. To prevent dramatic population shifts towards virulence, rendering host resistance all but useless, population monitoring and a dynamic

control strategy must be in place to counteract the negative effects of selection pressure.

Introduction

Potato late blight, one of the world's most devastating plant diseases in potato and tomato, is caused by the oomycete *Phytophthora infestans*. In the past, potato late blight led to e.g. the Great Irish - and Continental Famine (Zadoks, 2008). Currently potato late blight still is the most important disease in potato cultivation, traditionally controlled by highly frequent (calendar based) fungicide applications (Cooke et al., 2011) supported by preventative cultural measures such as crop rotation, the use of healthy seeds and the timely destruction of primary sources of inoculum. Nevertheless potato late blight remains responsible for an estimated annual economic loss of M€ 1000 on the 6 Mha of potato grown in the EU (Haverkort et al., 2008).

Host resistance and subsequent cultivation of potato late blight resistant potato cultivars is the most (cost) effective and environmentally friendly way to control potato late blight (Schepers et al., 2009). Currently however, cultivation of resistant cultivars is very limited due to an overwhelming demand for a limited number of commercially successful but highly late blight susceptible cultivars. In addition, potato breeding is complex and time consuming (Rietman et al., 2012) mostly due to the tetraploid nature of the crop. It was also repeatedly shown that resistance gene (R gene) mediated host resistance was easily overcome by the highly adaptive pathogen *P. infestans* (e.g. Black et al., 1953, Fry 2008; Haas et al. 2009; McDonald and Linde 2002), especially if resistance is based on a single R gene. The origin of the adaptive capability of *P. infestans* was shown to reside in the *P. infestans* genome in combination with its high reproductive capacity (Haas & Kamoun et al., 2009). As a result, adaptation is "the Phytophthora infestans way of life" resulting in R genes being overcome (e.g. Black et al., 1953, Fry 2008; Haas et al. 2009; McDonald and Linde, 2002), resistance to active ingredients of fungicides (e.g. Dowley and O'Sullivan, 1981) and increased aggressiveness (e.g. Flier and Turkensteen, 1999). The net results are the often dramatic and sudden population changes such as those described by Drenth et al. (1993), Cooke et al. (2012) and Fry et al. (2013).

Recently developed GM technology overcomes some of the disadvantages of conventional breeding with respect to breeding for resistance to potato late blight: Stacking R genes in popular commercial potato varieties in a fraction of the time required for conventional breeding while the cultivar characteristics remain the same was successfully demonstrated

within the DuRPh project (Haverkort et al., 2016).

It is however to be expected that (large scale) cultivation of (GM) resistant potato cultivars leads to selection pressure on the pathogen population for virulent genotypes. Within the DuRPh project a tailor-made potato late blight control strategy based on resistant cultivars aimed to mitigate the effects of selection pressure using a low input fungicide spray program (Haverkort et al., 2016). This strategy was also successfully deployed within the AMIGA project (AMIGA deliverables 8.3 and 8.6). For this purpose, five large scale field trials, containing a susceptible potato cultivar Desiree and two resistant potato cultivars/clones, were carried out in Ireland and the Netherlands. In order to quantify selection pressure, *P. infestans* was sampled in and around these field trials. Infections in the field trials were of natural origin and therefore represent the local *P. infestans* population. *P. infestans* sampled in the fields was genetically characterised using the standardized 12-plex Euroblight SSR set (Li et al., 2013). The resulting genotypic data were analysed for impact of resistance on composition of the local *P. infestans* population.

Materials and Methods

Field trials

Field trials are fully described in AMIGA DL 8.3 but they are summarized below:

Five field trials were carried out, two in the Netherlands in 2013 and 2014 (Valthermond, GPS coordinates 52.873828°, 6.942644) and three in Ireland in 2013, 2014 and 2015 (Oak Park, Carlow, GPS coordinates; 52.8560667, -6.9121167). Trials were carried out under permit IM10-006 for the Netherlands and in Ireland the trials were licensed by the Environmental Protection Agency as per Notification No. B/IE/12/01.

Two commercial potato cultivars and one potato clone were used in the field trials: the conventionally bred cultivar Desiree (highly susceptible to potato late blight), the conventionally bred cultivar Sarpo Mira (highly resistant to potato late blight) and the "Desiree based", cis-genically modified (Jacobsen and Schouten, 2007), highly resistant, *Rpi-Vnt1.1* containing clone A15-031 (described in detail in Haesaert et al., 2015). Sarpo Mira is reported to contain the R3a, R3b, R4, *Rpi-Smira1* and *Rpi-Smira2* potato late blight resistance genes (Rietman et al., 2012).

IPM field trials were laid out as randomized block experiments including the three potato genotypes mentioned above, three potato late blight control strategies (unsprayed control, weekly spray schedule and a "next level IPM control strategy", (see AMIGA DL8.3)) and seven replicates.

Monitoring plots, located outside the trials themselves, served the purpose of monitoring the local *P. infestans* population for adaptation against the R-genes deployed in the field trial and thus quantification of selection pressure. Trials in Valthermond were surrounded by ten monitoring plots whereas the trials at Oak Park were surrounded by 11 monitoring plots in 2014 and 2015. Monitoring was not possible for Ireland in 2013 due to the unavailability of seed of the conventional clone RH06-975-8 containing *Rpi-vnt1.3*. Each monitoring plot contained 6 (NL) or 3 (IE) plants of Desiree, Sarpo Mira and the conventional clone RH06-975-8. RH06-975-8 was used as a conventional substitute for the Cisgenic *Rpi-Vnt1.1* containing clone A15-031. Monitoring plots were not sprayed with crop protection products

but at times (e.g. Ireland 2014) received additional irrigation. From emergence onwards, these plots were monitored for infection on a weekly basis. When infection was found, this was quantified as the number of lesions and the percentage destroyed foliage (severity). Also a *P. infestans* DNA sample was taken by pressing the sporulating part of a single lesion on an FTA card (Whatman International Ltd, Maidstone, United Kingdom). A maximum of 4 DNA samples was taken per potato genotype, monitoring plot and year. Infections occurring in the field trials were quantified and sampled in the same manner as done for the monitoring plots. However, lesion counts were omitted as it was found to be impractical. FTA cards were collected and stored at room temperature until further molecular analysis.

Genotypic characterisation

The Euroblight standardized set of twelve microsatellite markers was used for genotypic characterisation of the *P. infestans* samples (Knapova and Gisi 2002; Lees et al. 2006; Li et al. 2010; Li et al. 2013). Amplification of the SSR markers was carried out as described by Li et al. (2010). Capillary electrophoresis was carried out on an automated ABI-3130xl sequencer (Ireland 2014+2015) using a 16-capillary array (36 cm) or an automated 48-capillary array (36 cm) ABI 3730 according to the manufacturer's instructions. SSR allele scoring and sizing was done using GeneMapper version 3.7 (Applied Biosystems).

Data analysis

For the purpose of this analysis, results from the monitoring plots and the field trials were pooled since no monitoring plot samples were available from the Irish field site. The genetic distance between field samples was determined and visualized using the R package POPPR 2.2.1 (Kamvar et al., 2014, Kamvar et al., 2015). SSR data was formatted as a GenAlEx csv file in Excel 2010 (© 2010 Microsoft Corporation) and then imported into R as a genclone object. Genetic distances were estimated using Bruvo distance (Bruvo et al., 2004). Subsequently a minimal spanning network (msn) was drawn based on these bruvo distance estimates. Field samples were assigned to groups based on the year, country of origin and host genotype they were sampled from. Each group was assigned a unique colour with similar colours for

the same host-genotypes irrespective of country of origin. Sub-setting of data was performed in order to investigate selection pressure from the resistant potato genotypes on *P. infestans* genotypes found. Nodes represent the number of samples with a minimum of 1. Larger nodes therefore represent multiple field samples with identical multi-locus genotypes (mlg). The number inside the node indicates the number of samples represented. The correlation between node size and number of represented samples has been modified by base logarithm 1.3 in order to prevent the larger nodes obscuring results in surrounding nodes. For the field samples from The Netherlands a genetic distance cut-off of 0.3 was used. This means isolates sharing less than 70% genetic similarity (based on SSR alleles) will not be connected in the minimal spanning network. For Ireland a cut-off of 0.1 was set, forcing samples sharing less than 90% genetic similarity into different groups, highlighting groups of samples with a similar clonal mlg. The strict cut-off value for Ireland allows to assert the clonal nature of the population. The higher cut-off value for the Netherlands allows to investigate whether groups of mlg's e.g. share a certain virulence profile. Within the msn, genetic distance is correlated with line thickness: wide black lines indicate lower genetic distance whereas thin grey lines indicate greater genetic distance. The maximum value for genetic distance connected by lines is set by the cut-off value discussed above.

Results

From the five field trials, a total of 360 samples were taken, genotypically characterised and analysed. The origin of these samples is given in Table 1. Irish samples were only sampled from the AMIGA trial itself.

Country / location effects

Table 1 shows *P. infestans* was not found on Vnt1 containing plant material in Ireland. This in contrast to the Netherlands where *P. infestans* was found both on A15-031 in the field trial and on the conventional Vnt1 clone in the monitoring plots. In addition, Figure 1 demonstrates a much higher level of genetic variation present in the *P. infestans* population in Valthermond (the NL) as compared to the *P. infestans* population in Carlow (Ireland). The pathogen population at Carlow is primarily made up of three well known clonal lineages: EU_13_A2 (Blue13), EU_6_A1 (Pink6) and EU_8_A1. The pathogen population at Valthermond is primarily made up of individuals outside the well-known clonal lineages apart from 16 samples that likely belong to the EU_13_A2 clonal lineage. The *P. infestans* populations in both countries are therefore genetically very different.

Table 1. Origin of *P. infestans* DNA samples taken in and around field trials during the AMIGA project.

Country	Year	Type field	Potato genotype	Nr of DNA samples obtained	Total per year	Total per location
Ireland	2013	Trial	Desiree	48		
			Vnt1 clone	0		
			Sarpo Mira	44	92	
	2014	Trial	Desiree	45		
			Vnt1 clone	0		
			Sarpo Mira	17	62	
	2015	Trial	Desiree	43		
			Vnt1 clone	0		
			Sarpo Mira	0	43	197
Netherlands	2013	Monitoring	Desiree	27		
			Vnt1 clone	0		
			Sarpo Mira	7		
		Trial	A15-31	34		
			Desiree	37		
			Sarpo Mira	12	117	
	2014	Monitoring	Desiree	29		
			Vnt1 clone	7		
			Sarpo Mira	10	46	163

From Figure 1 it can be concluded that almost all the different *P. infestans* genotypes found are capable of infecting susceptible cultivar Desiree (light and dark green in Figure 1). However, only specific *P. infestans* mlgs (clonal variants for IE) are capable of infecting Sarpo Mira (orange and yellow) and/or Vnt1 containing plant material (shades of red). Interestingly, for samples from Ireland it was found that only certain sub-variants of EU_8_A1 were found on Sarpo Mira plants. This could indicate that Sarpo Mira exerts selection pressure against specific EU_8_A1 isolates.

Year Effects

Carlow: 2013 and 2015 show a more or less equal distribution of clonal lines EU_8_A1 and E_13_A2 on Desiree (Figure 2). In 2014 the population was mostly dominated by EU_8_A1 with a small contribution from EU_13_A2. The limited number of EU_6_A1 was only found (on Desiree) in 2014.

Valthermond: *P. infestans* populations on all three potato cultivars for 2013 and 2014 are related but different with very few overlapping genotypes between years and a relatively small contribution of the well-known clonal lineages (Figure 2). Based on the results on Desiree, the 2014 population seems to be slightly more "clonal" as compared to the 2013 population.

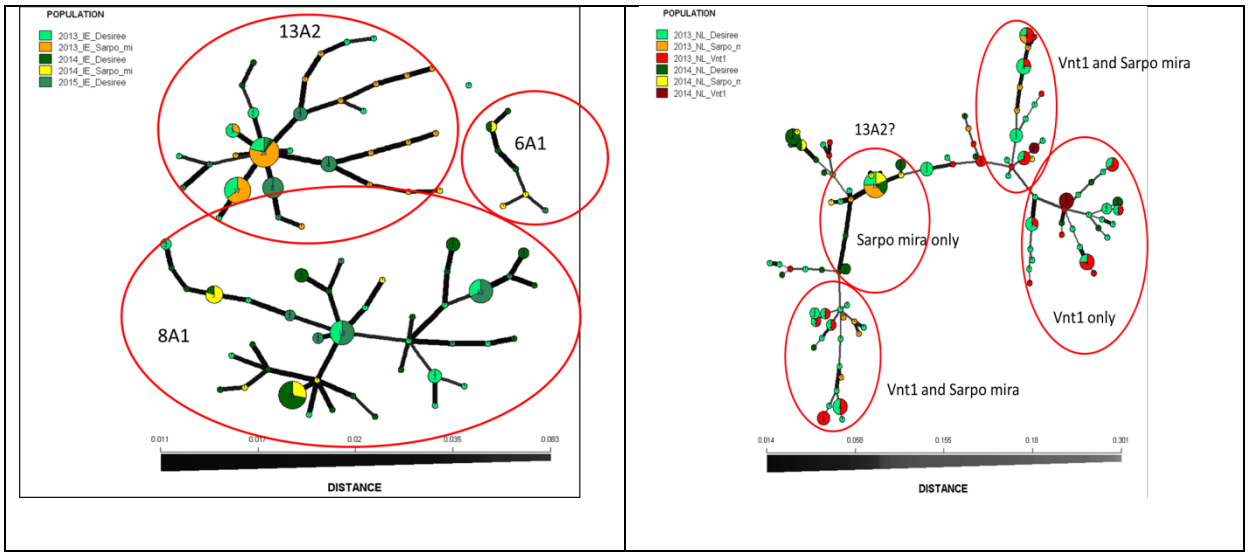


Figure 1. Genetic distance between samples from Ireland (left panel) taken in 2013, 2014 and 2015 and The Netherlands (right panel) taken in 2013 and 2014 as an indication for the genetic variation among *P. infestans* samples in and around the two AMIGA field trials. Numbers in each node indicate the number of identical SSR multi-locus genotypes represented. Distance indicates the genetic distance represented by the lines connecting nodes in the network.

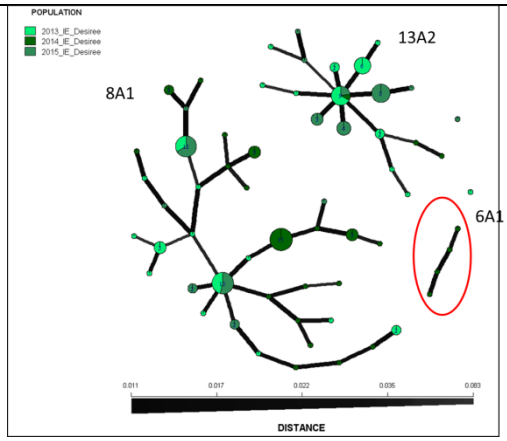
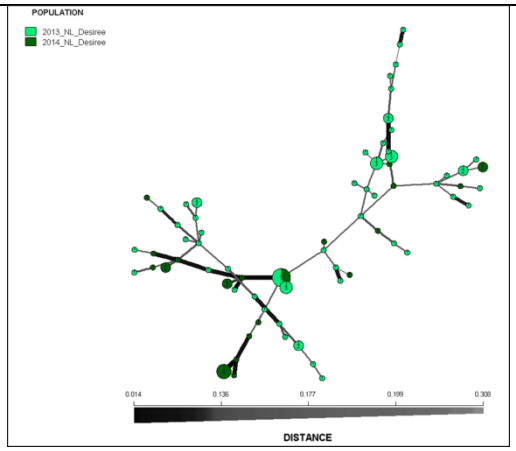
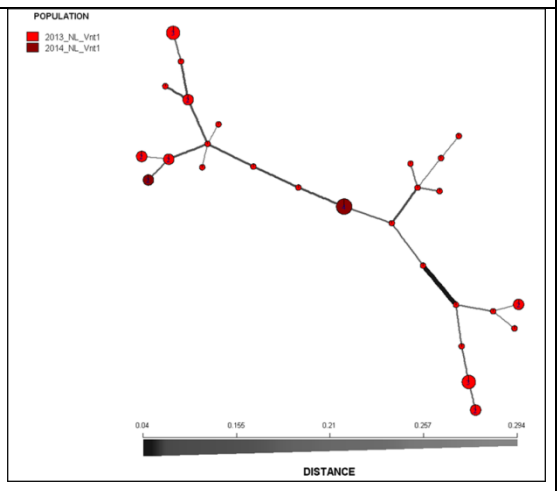
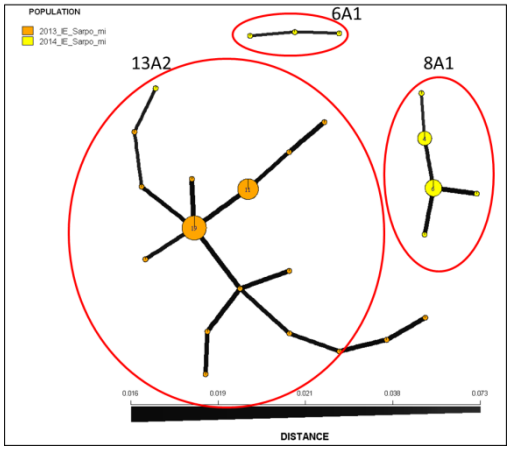
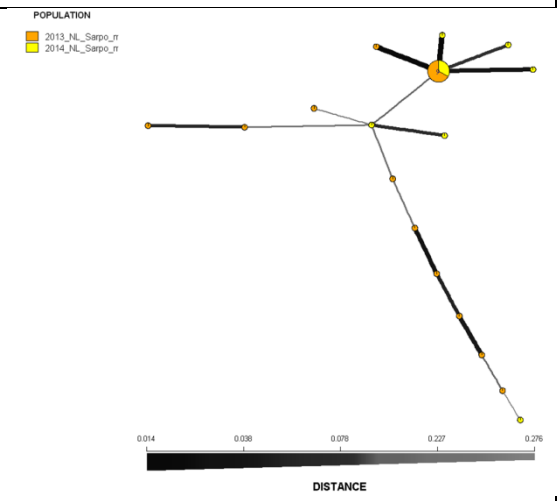
	Carlow (IE)	Valthermond (NL)
Desiree		
Vnt1		
Sarpo Mira		

Figure 2. Genetic variation per potato clone, location and year. Numbers in each node indicate the number of identical SSR multi-locus genotypes represented. Distance indicates the genetic distance represented by the lines connecting nodes in the network.

Cultivar effects

Desiree : At both locations, Desiree is infected by almost all *P. infestans* genotypes (in blue, top row in Figure 3), demonstrating a good representation of almost all genotypes found or on this susceptible cultivar.

Sarpo Mira: In Carlow, this cultivar is infected by sub-variants of the well-known clonal lines (EU_13_A2, EU_8_A1 and EU_6_A1). In Valthermond EU_13_A2 variants also infect Sarpo Mira. In addition other, more genetically distinct, *P. infestans* genotypes also occasionally infect Sarpo Mira in Valthermond.

Vnt1 containing plant material: Vnt1 containing plant material is only infected in Valthermond, the Netherlands (Figure 3). This is very likely due to the large genetic variation contained in the local pathogen population as a result of an active sexual cycle and regular oospore contributions to main-crop infections. Most Vnt1 infecting *P. infestans* genotypes are more genetically distinct and outside the well-known clonal lines described earlier. If we include the results from Figure 1, Vnt1 infecting *P. infestans* genotypes can be grouped in at least three groups, with or without the additional capability to infect Sarpo Mira. Based on the data presented it can be concluded that virulence against Vnt1 can be found amongst a wide range of genetically distinct *P. infestans* genotypes. It is therefore likely that this ability/virulence can spread quickly throughout sexually recombining *P. infestans* populations such as those in The Netherlands. In contrast none of the traditional clonal multi-locus genotypes were found on Vnt1 containing plants.

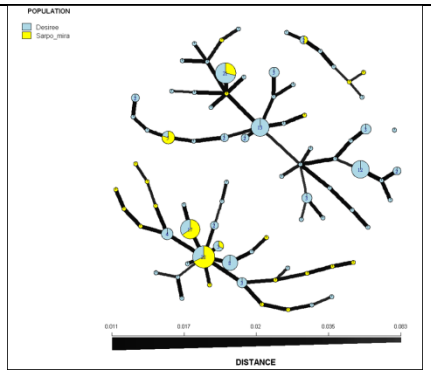
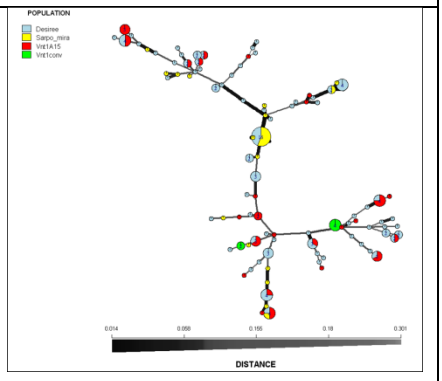
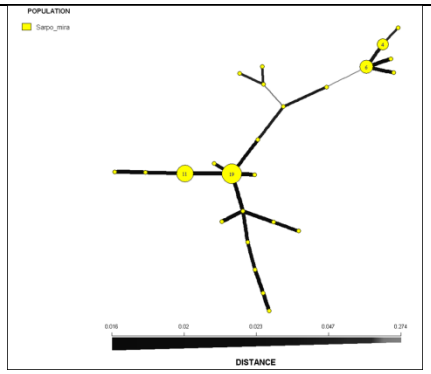
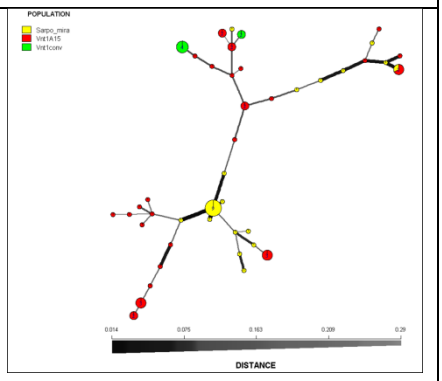
	Carlow	Valthermond
All Cultivars		
Resistant cultivars		

Figure 3. Genetic variation of the local *P. infestans* population on Desiree, Sarpo Mira and Vnt1 containing plant material. Numbers in each node indicate the number of identical SSR multi-locus genotypes represented. Distance indicates the genetic distance represented by the lines connecting nodes in the network.

Conventional Vnt1 versus GM Vnt1 containing plant material

Figure 4 gives the genetic distances for *P. infestans* samples taken from the cisgenic *Rpi-Vnt1.1* containing potato line A15-031 and *P. infestans* samples taken from conventional *Rpi-Vnt1.3* containing potato clone RH06-975-8. Despite the fact that *Rpi-Vnt1.1* and *Rpi-Vnt1.3* are reported to share the same resistance spectrum (Pel et al., 2009), conventional-Vnt1 infecting *P. infestans* genotypes are genetically distinct from GM-Vnt1 infecting *P. infestans* (green- versus red nodes in Figure 4). Also within both groups only a few identical mlgs are found. This is a direct indication for selection pressure in general and selection of different *P. infestans* genotypes by the GM and non-GM, Vnt1 containing potato clones. All 34 samples found on A15-031 are however from 2013 whereas all 7 samples from the conventional Vnt1 clone are from 2014. In this case, the "year factor" is thus confounded with the factor "genotype of origin" resulting in an inconclusive answer on the question whether a genetically modified potato selects for distinct *P. infestans* genotypes when compared to

conventionally bred potato varieties. This is the case even more if we consider the differences between the 2013 and 2014 *P. infestans* populations in Valthermond reported above. Last, but not least, this finding might also be an indication of the presence of other R genes in the conventional Vnt1 containing potato clone.

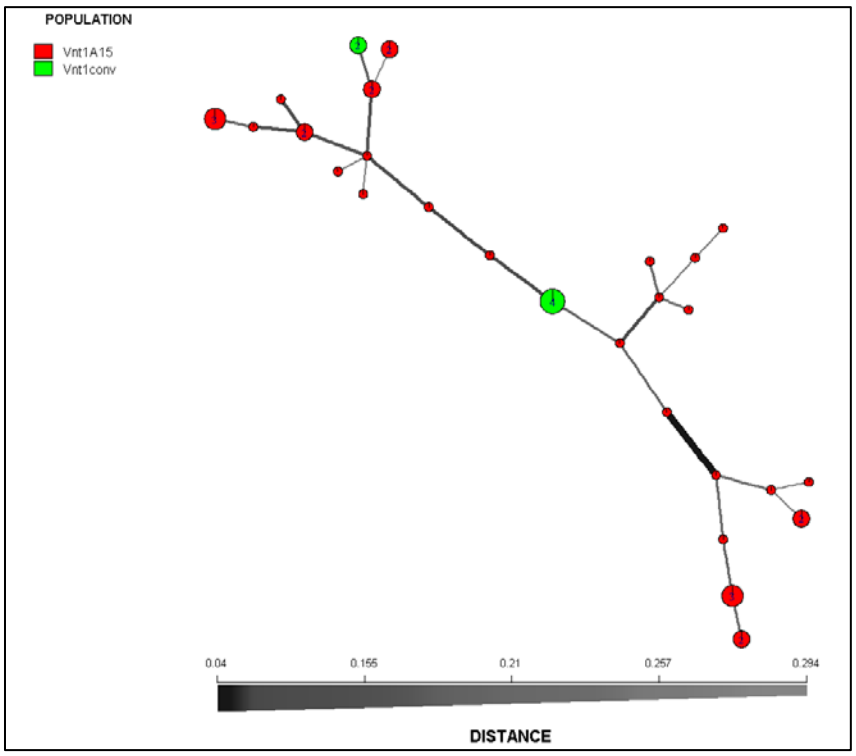


Figure 4. Genetic distance between P. infestans samples taken from the cisgenic potato line A15-031 (red) compared to P. infestans samples taken from conventional potato clone Vnt1 (green). Numbers in each node indicate the number of identical SSR multi-locus genotypes represented. Distance indicates the genetic distance represented by the lines connecting nodes in the network.

Discussion

The purpose of AMIGA DL 8.4 is to deliver quantitative data on the impact of host resistance genes on the population dynamics of *P. infestans*. Selection pressure causes shifts in the pathogen population towards e.g. virulence for specific (combinations of) R-gene(s). Occurrence of selection pressure indicates the critical need for continuous pathogen population monitoring and a dynamic control strategy to counteract any negative effects of selection pressure.

For this purpose *P. infestans* was sampled and genetically characterised in and around the field trials performed in Ireland and the Netherlands. The results (Figure 1) indicate clear differences between the local pathogen populations in Carlow (Ireland) and Valthermond (the Netherlands). The Carlow population is primarily made up of a few well known clonal lines whereas the Valthermond population contains greater genetic variation and is primarily made up of genetically distinct isolates plus contributions from clonal line EU_13_A2. Over the three years, the composition of the Carlow population was more or less stable with varying contributions of the same clonal lines. In Valthermond, EU_13_A2 was present in all three years but the majority of the pathogen population was genetically distinct between both years (Figure 2). The difference in genetic variation contained in both populations is very likely due to the effect of an active sexual cycle in the *P. infestans* populations in or around the Valthermond site. The highly undesirable result, as illustrated, is that R genes and thus host resistance in general is more likely to be overcome by pathogen populations harbouring high(er) levels of genetic variation.

Susceptible cultivar Desiree hosted almost all of the *P. infestans* genotypes found in Carlow and Valthermond. Resistant cultivar Sarpo Mira and the resistant, Desiree based, cisgenically derived A15-031 both projected significant selection pressure on the pathogen population (e.g. Figure 3). In Carlow, Sarpo Mira was infected by a limited number of sub-variants of clonal line EU_13_A2, EU_6_A1 and EU_8_A1. In Valthermond, Sarpo Mira was also infected by sub-variants of EU_13_A2 plus a range of genetically distinct isolates. Vnt1 containing potato clones were not infected in Carlow which is consistent with published evidence that EU_13_A2 does not overcome the Vnt1 gene (Cooke et al., 2012). In Valthermond they were infected by a range of genetically distinct pathogen genotypes indicating the potential for rapid changes towards a virulent pathogen population once the R gene is used on larger

acreages.

An attempt to investigate specific selection pressure exerted by GM crops versus non-GM crops remained inconclusive. Insufficient samples were successfully genotyped on the conventional Vnt1 clone, factors “year” and “origin” were confounded: all conventional Vnt1 samples came from 2014 whereas all GM Vnt1 samples came from 2013 and the pathogen populations in Valthermond demonstrate a large variability between years. Last, but not least, this finding might also be an indication of the presence of other R genes in the conventional Vnt1 containing potato clone.

Overall, it is clear that cultivation of resistant potato exerts significant selection pressure on local *P. infestans* populations. To prevent selection driven, dramatic pathogen population shifts, rendering host resistance all but useless, population monitoring and a dynamic control strategy must be in place to counteract the negative effects of selection pressure. Host resistance breaking traits would need to be countered by careful deployment of resistance genes in space and time (Skelsey et al 2010) in combination with a low input spray strategy when necessary (AMIGA DL 8.3 + 8.6).

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