Resistance Based Integrated Pest Management Strategy for *Globodera rostochiensis* and *Globodera pallida* in Potato Cropping Systems

Dissertation

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List of Abbreviations and Acronyms

Ar	Allelic richness
Avr	Avirulence
CABI	Centre for Agriculture and Bioscience International
CO1	Cytochrome oxidase 1
DAI	Days after inoculation
Cv.	Cultivar
DD	Degree-days
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DPI	Day post inoculation
EPPO	European and Mediterranean Plant Protection Organization
FCA	Factorial correspondence analysis
F_{IS}	Inbreeding coefficient
F_{ST}	Fixation index
g	Gram
H_{nb}	Genetic diversity
Hr(s)	Hour (s)
HWE	Hardy-Weinberg equilibrium
IPM	Integrated pest management
J2	Second stage juvenile
J3	Third stage juvenile
J4	Forth stage juvenile
K	cluster
LSU	Long sub-unit
MAS	Mass assisted selection
min	Minutes
ml	millilitre
mM	Millimolar
n	Sample size
PCN	Potato cyst nematodes
PCR	Polymerase chain reaction
P_f	Final population density
pH	Potential of hydrogen
P_i	Initial population density
PPN	Plant parasitic nematodes
PRD	Potato root diffusate
QTLs	Quantitative trait loci
RAPD-PCR	Restriction amplified polymorphic DNA-PCR
rDNA	Ribosomal deoxyribonucleic acid

List of Abbreviations

R_f	Reproduction factor
RFLP	Restriction fragment length polymorphism
RKN	Root knot nematode
rpm	Revolution per minute
R_s	Relative susceptibility
s	second
SE	Standard error
TC	Tissue culture
Tris-HCL	Tris hydrochloride
µl	Microlitre
wv	Weight per volume
µm	micrometre
$ZnCl_2$	Zinc chloride

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Chapter 1: General Introduction

1.1 Introduction to Potato Cyst Nematodes

The potato cyst nematodes (PCN), *Globodera pallida* (Stone) and *Globodera rostochiensis* (Wall.) are important pathogens of potato (*Solanum tuberosum*). They originated in the Andes mountains of South America (Mai, 1977; Grenier *et al*., 2010) where they co-evolved with potatoes for thousands of years (Stone, 1979). PCN were introduced into Europe in the mid-19th century probably with breeding materials (Turner and Evans 1998), but first discovered towards the end of 19th century in the Rostock region of Germany (Moens et al., 2018). Europe served as the second source of introduction to other parts of the world (Turner & Evans, 1998; Plantard et al., 2008). To date, PCNs occur in many potato growing countries all over the world (CABI, 2019). For a long time, they were considered temperate pests, but have gradually spread to subtropical and tropical countries where potatoes are grown (Greco, 1993; Alonso *et al*., 2011; Moens *et al*., 2018). *Globodera pallida* has been reported in over 55 countries, *G. rostochiensis* is present in almost 100 countries worldwide ((CABI, 2019), Fig. 1.1). In 2015, *G. rostochiensis* was first reported in Kenya (Mwangi et al., 2015), followed by *G. pallida* three years later (Mburu et al., 2018).

PCN move only short distances in their life time (Wallace, 1968). They disperse mainly through movement of infested potato tubers, contaminated machinery, foot wares and clothes, among others (Mai, 1977; Brodie, 1993; Anonymous, 2017a). Short distance dispersal, such as within or between potato fields, is by run-off water, dust blown by wind, contaminated farm equipment, movement of humans and animals (CABI, 2019).

PCN cause high losses in terms of yield reduction, reduced quality and associated costs of nematode management. The global losses are difficult to estimate (Singh et al., 2013), but in Europe alone, approximately 9% of potato yield is lost to PCN annually (Turner and Subbotin, 2013). At the field scale, high nematode infestation can lead to total crop failure. Due to potential damage and difficult management, PCN are worldwide regulated as quarantine organisms (Anonymous, 2017a; CABI, 2019). This affects local and international trade with potatoes as well as other tuber crops (Faggian et al., 2012; Blacket et al., 2019).

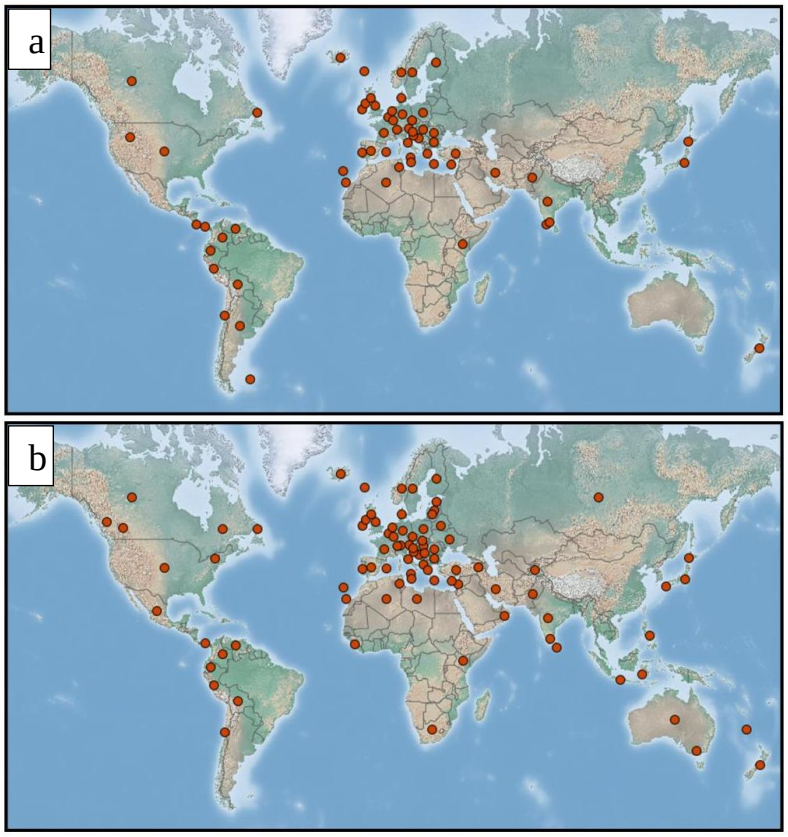

Figure 1.1 World map indicating the global distribution (in red dots) of *G. pallida* (a) and *G. rostochiensis* (b) (Source: (CABI, 2019)).

1.2 Genetic Diversity

Co-evolution between PCN and potatoes resulted in high genetic diversity within and between the species (Rausher, 2001). Until the 1920s, *G. rostochiensis* and *G. pallida* were considered one species, *Heterodera rostochiensis* (Fleming and Powers, 1998). In the early 1950s, the first resistance gene to PCN (*H1* gene) was identified and introgressed into commercial potato cultivars (Ellenby, 1952). Shortly after, populations emerged that were able to overcome this resistance and they were found to be morphologically different. It therefore appeared that *Heterodera rostochiensis* comprised of two different nematode groups distinguishable by the colour of the maturing females and specific morphological differences. This led to the description of the second PCN species, *Heterodera pallida*, now known as *Globodera pallida* (Stone, 1972). Later, *Heterodera rostochiensis* was renamed *Globodera rostochiensis*. Further deployment of resistant cultivars revealed more differences within each of the two species. There were several virulent

populations in both species that differed in their reproduction on different resistance genes (Fleming & Powers, 1998). For instance, some *G. pallida* populations were able to reproduce on cultivars carrying the *H2* gene which conferred resistance to *G. pallida* (Dunnet, 1961). This led to the establishment of the pathotyping scheme (Kort et al., 1977) in which populations with common (a)virulence genes were grouped together.

Kort et al. (1977) proposed a scheme of pathotypes to differentiate between populations of the same species that were able to reproduce on different resistant cultivars. The designation of a population into a pathotype group was based on the reproduction rate calculated as the ratio of the final nematode density to the initial nematode density inoculated (P_f/P_i) on a set of differential potato clones (Table 1.1). A clone with a $P_f/P_i > 1$ was considered susceptible while $P_f/P_i < 1$ was considered resistant (Kort et al., 1977). Using this scheme, *G. rostochiensis* was grouped into five pathotypes (Ro1, Ro2, Ro3, Ro4, & Ro5), whereas *G. pallida* was grouped into three pathotypes (Pa1, Pa2 & Pa3) (Kort et al., 1977). However, some nematode populations, in particular belonging to G. *pallida*, were too heterogeneous to fit into the scheme. The pathotyping system had many practical applications in PCN management, but was criticized for relying on potato clones with polygenic resistance (Mugniéry et al., 1989). Such clones could be attacked by nematodes with a continuum of virulence that could not fit in the pathotype scheme (Trudgill, 1985). Also, the scheme was considered faulty for using the arbitrary value of $P_f/P_i = 1$ to delimit resistance (Trudgill, 1985; Mugniéry et al., 1989; Nijboer & Parlevliet, 1990; Greco et at., 2007). To improve the scheme, Nijboer & Parlevliet (1990) prosed two pathotypes in *G. pallida* (Pa1 & Pa2/3), merging Pa2 and Pa3 which did not seem to differ in virulence. Similarly, they proposed three virulence groups for *G. rostochiensis* Ro1 (Ro1 and Ro4), Ro3 (Ro2 and Ro3) and Ro5.
In addition, it was proposed that the resistance of a clone should be estimated by relating the reproduction of the nematodes on the clone with the reproduction on a reference susceptible cultivar in order to quantify the proportion of virulent nematodes in a population (Trudgill, 1985). Although, the pathotyping scheme has weaknesses, it is still an important tool for breeders and researcher working with PCN (Fleming & Powers, 1998; Blok et al., 2018).

Table 1.1 International pathotype scheme for *G. rostochiensis* and *G. pallida* on *Solanum* spp. according to Kort et al. (1977).

Cultivar/clone	Pathotypes*							
	Ro1	**Ro2**	**Ro3**	**Ro4**	**Ro5**	**Pa1**	**Pa2**	**Pa3**
S. tuberosum ssp. *tuberosum* 'Desiree'	+	+	+	+	+	+	+	+
S. tuberosum ssp. *andigena* (*H1*)'Laura'	−	+	+	−	+	+	+	+
S. kurtzianum-hybr KTT/60.21.19	−	−	±	±	±	+	+	+
S. vernei-hybr GLKS.58.1642.4	−	−	−	+	+	+	+	+
S. vernei-hybr $(VT^n)^2$ 62.33.3	−	−	−	−	±	−	−	+
S. vernei-hybr 65. 346.19	−	−	−	−	+	+	+	+
Solanum multidissectum P55/7	+	+	+	+	+	−	+	+

*"+" = susceptibility ($P_f/P_i > 1$); "-" = resistance ($P_f/P_i < 1$); "±" = partial or uncertain

Understanding the diversity of nematode populations is important for predicting and therefore prolonging the efficacy and durability of the available plant resistance (McDonald & Linde, 2002; Gautier et al., 2019). The information helps to reconstruct the source and route of introduction of a nematode population and therefore prevents further spread (Plantard & Porte, 2004; Blacket et al., 2019). In addition, such information is vital when searching for resistance genes to a particular nematode population (Grenier et al., 2010).

There are several molecular markers to study the genetic diversity of PCN populations. For example, restriction fragment length polymorphism (RFLP) markers have been used to differentiate populations of *G. pallida* (Schnick et al., 1990). Randomly amplified polymorphic DNA (RAPD-PCR) was used by Bendezu et al. (1998) to study the inter- and intragenic variability between the European and South American populations of PCN. On their part, Nuaima et al. (2019) used denaturing gradient gel electrophoresis (DGGE) fingerprinting to assess variations within and among different PCN populations. Through sequencing of ITS regions of rDNA, it is possible to create a phylogeny of nematode populations (Subbotin et al., 2001), whereas microsatellite DNA markers are powerful tools for investigating the origin of nematode populations (Plantard et al., 2008), and to study the genetic diversity and population structure of PCN populations (Boucher et al., 2013; Blacket et al., 2019).

1.3 Biology

Potato cyst nematodes are sedentary endoparasites that infest and complete their life cycle in the roots of a limited number of hosts (Sullivan et al., 2007). Both PCN species reproduce sexually and eggs are secured in an encapsulated structure called cyst (Cook & Noel, 2002). The life cycle of cyst nematodes has four juvenile stages followed by the adult stage. The first stage juvenile (J1) is formed within the egg (Fig. 1.2a) and it moults into the second-stage juvenile (J2) within the

egg. The J2 remain in a dormant state until the environment is conducive for hatching. Hatching is triggered by chemical stimuli present in the root leachate of compatible hosts. During hatching, the J2 uses its stylet to probe and cut a slit through the eggs and emerges (Fig. 1.2b). The J2 is the active and infective stage in the nematode life cycle. It moves into the surrounding soil and locates the host root. Using the stylet, the J2 pierces through the epidermal cells of the root near the root tip and penetrates (Fig. 1.2c). The juvenile moves intracellularly towards the pericycle and establishes a permanent feeding site. The J2s inject saliva via the stylet and induce the formation of a syncytium which continuously nourishes the nematode as it develops (Moens *et al*., 2018). Subsequently, they lose mobility and become sedentary. They feed continuously and undergo three consecutive moults into third (J3, Fig. 1.2d), then to fourth stage juvenile (J4, Fig. 1.2 e & g) before moulting into either male (Fig. 1.2f) or female nematodes (Fig. 1.2h). The male is vermiform in shape and leaves the root and mates with the saccate female. Finally, the fertilized female matures and develops eggs within its body. Once eggs have developed the female dies, the body tans and dries to form a brown cyst (Fig 1.2i). Each cyst contains approx. 200 to 500 eggs. The cyst is a survival structure and can persist in the soil for up to 30 years (Turner & Subbotin, 2013; Moens *et al*., 2018). The life cycle of the nematode takes between 6 to 10 weeks depending on the temperature (Mugniéry, 1978; Philis, 1980; Ebrahimi et al., 2014). In the temperate regions, PCN have one generation per year. However, the number of generations can be higher in different cropping systems particularly in tropical and subtropical regions (Philis, 1980; Greco et al., 1988; Moens et al., 2018).

Potato cyst nematodes have a narrow host range, they infest and complete their life cycle in the roots of potato, eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*) and other plants of the Solanaceous family (Sullivan et al., 2007). The symptoms induced by PCN infestation resemble those caused by other biotic or abiotic stress factors (Mulder & Van Der Wal, 1997). They include poor growth, reduced ground cover, yellowing of leaves, premature haulm senescence, wilting, stunted growth, reduced number and size of the tubers or complete crop failure under severe infestation, among others (Mulder & Van Der Wal, 1997; Moens *et al*., 2018).

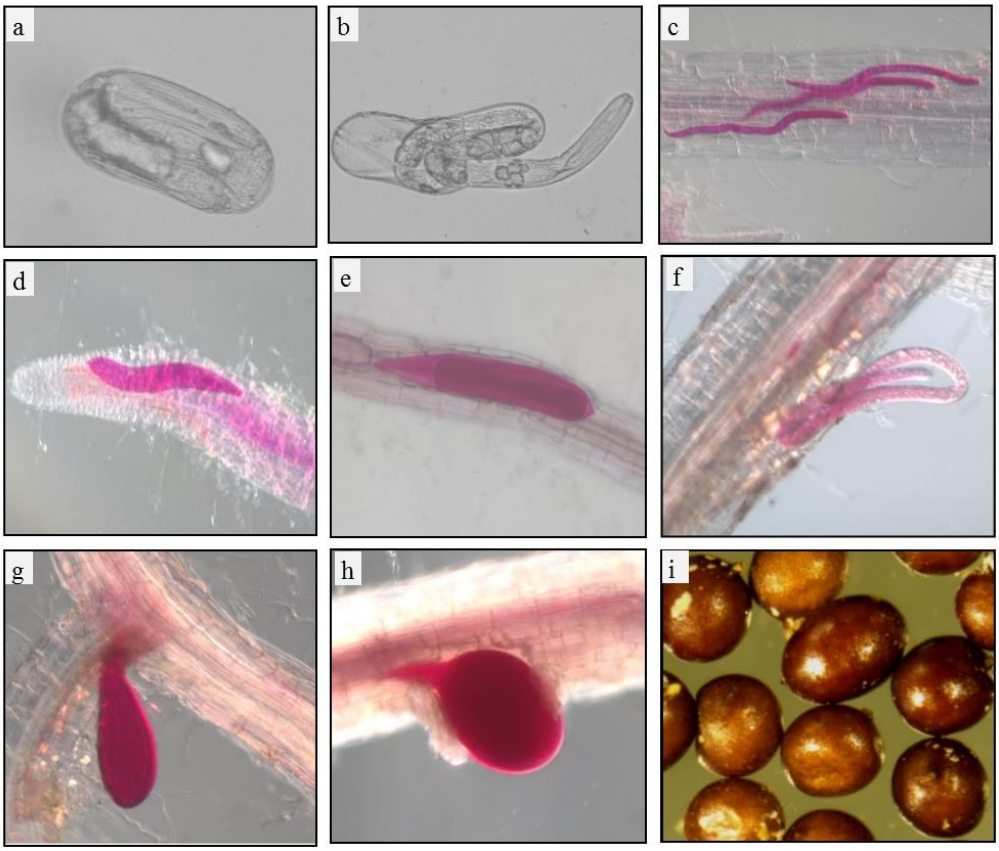

Figure 1.2 Representative stages of PCN life cycle; (a) Egg containing J1; (b) J2 hatching from the egg; (c) J2 in the root system; (d) J3 in the root; (e) J4 male in the root; (i) Adult male exiting the root; (g) J4 female; (h) Adult female; (i) Cyst. Photos: James Mwangi, 2019.

1.4 Nematode-Host Interaction

Potato cyst nematodes have a repertoire of effectors, i.e. proteins and other small molecules secreted by pathogens that alter host-cell structures and functions during infection (Hogenhout et al., 2009). They use these effectors to overcome the host's resistance, supress immune response and induce the formation and maintenance of the syncytium (Jones & Mitchum, 2018). In response, plants have developed a robust defence system to evade nematode infestation (Goverse & Smant, 2018). The interaction with hosts possessing a specific resistance gene leads to the recognition of specific elicitors or effectors produced by specific avirulence genes in the nematode. Using host-specific receptors coded by the resistance genes, plants are able to detect the nematode effectors and to respond by activating a cascade of defence responses to restrict the development of the nematode in the roots (Goverse & Smant, 2018). This form of interaction is usually explained by the gene-for-gene hypothesis where the resistance gene in the host has a

corresponding avirulent (*Avr*) gene in the nematode (Jones, 1974). The nematode-host interaction results in resistance, if the resistance gene is able to detect the *Avr* gene products of the nematode (Trudgill, 1991). Otherwise, the nematode is able to infect and reproduce proportional to the level of quantitative resistance in the host.

Individual nematodes, that are able to overcome the hosts' resistance and to complete their life cycle, are virulent (Trudgill, 1991). However, some hosts are able to restrict the reproduction of nematodes in them. Such hosts are resistant to that particular nematode population (Trudgill, 1991). Resistance genes do not offer immunity from attack by the nematodes, rather the resistance response in the host prevents the reproduction as opposed to susceptible hosts where the nematode is able to invade and reproduce.

As part of their defence system, plants may also have passive resistance in form of preformed structures that prevent entry of pathogens (Giebel, 1982). Preformed resistance includes physical, physiological and chemical barriers that are naturally present in the host (Giebel, 1982). To overcome this form of resistance, nematodes release effectors during penetration. For example, juveniles secrete enzymes such as cellulase and pectate lyase (Giebel, 1982) that degrade the cellulose and pectin in the cell wall to allow for penetration (Jones & Mitchum, 2018). Such effectors may trigger an active form of resistance, i.e. a post-infection response to the presence of the nematode. Plants produce secondary metabolites, salicylic acid, reactive oxygen species and β-1-4 endoglucanases that activate defence responses (Siddique et al., 2014). Some of these chemicals are responsible for hypersensitive reactions causing cell death (Giebel, 1982) and this affects nematode development in the roots.

1.4.1 Resistance Genes

A number of resistance genes against PCN have been identified and subsequently introgressed into potato cultivars (Bakker et al., 2006; Dalamu et al., 2012). They include several major genes (*R* genes) and quantitative trait loci (QTLs). *R* genes confer qualitative or monogenic resistance against specific PCN pathotype groups. For example, the *H1* gene was identified in *Solanum tuberosum* ssp. *andigena* CPC 1673 (Ellenby, 1952) and introgressed into commercial potato cultivars. This gene has successfully controlled *G. rostochiensis* Ro1 and Ro4 for the last half century (Bakker et al., 2006). Another major gene, *H2* gene, was identified in *Solanum multidissectum* (Dunnet, 1961) and it confers resistance to the Pa1 pathotype of *G. pallida*

(Bakker., 2006; Dalamu et al., 2012). Other *R* genes include *Gpa2* which confer resistance to *G. pallida* (Van Der Voort et al., 1997), G*roVI* and *Gro1-4*, which confer resistance to the Ro1 pathotype of *G. rostochiensis*. Breeding for PCN resistance with *R* genes is relatively durable and effective compared to QTLs (Bakker et al., 2006). Major genes offer complete resistance to the respective nematode pathotypes and the expression of the genes is generally little affected by genotype-environment interactions (Phillips, 1985).

Several QTLs have been mapped to different chromosomes in potato (Bakker et al., 2006; Dalamu et al., 2012). They confer a quantitative or polygenic form of resistance (Bakker et al., 2006) which is a partial resistance to some PCN pathotypes. The expression of QTLs is highly influenced by genotype-environment interactions. QTLs are difficult to introgress (van der Voort et al., 2000) and heterogeneous nematode populations can overcome QTLs within a few years of consistent use (Eoche-Bosy et al., 2017). QTLs have been mapped in genetic resources such as *Solanum spegazzinii, S. vernei, S. sparsipilum* and *S. multidissectum*, among others (Bakker *et al*., 2006; Dalamu *et al*., 2012). Four QTL, genes *Gro1*, *Gro1.2*, *Gro1.3* and *Gro1.4*, derived from *S. spegazzinii* confer resistance to *G. rostochiensis* while *Gpa (S. spegazzinii), Gpa4 (S. tuberosum* spp. *tuberosum), Gpa5* and G*pa6* derived from *S. vernei* confer resistance to *G. pallida* (Bakker et al., 2006; Dalamu et al., 2012).

The *H1* gene triggers a hypersensitive reaction characterized by necrosis and death of cortical cells surrounding the invading juvenile (Rice et al., 1985; Smant et al., 2018). This restricts the development of a syncytium (Moens et al., 2018). Consequently, the juvenile dies within the root tissue or emerges from the root (Giebel, 1982; Forrest et al., 1986). Some juveniles are able to survive with limited food resources, and, because sex in PCN is epigenetically determined (Trudgill, 1967), they develop into males that do not require further nourishment to survive (Giebel, 1982; Moens et al., 2018). In cultivars carrying the *Gpa2* gene, the formation of a syncytium is initiated, but the proliferation is arrested within a few days. The cells surrounding the syncytium become necrotic leading to the degeneration of the feeding structures. In this case, females form, but their development is arrested (Bakker et al., 2006).

1.4.2 Measuring Resistance/Virulence

Quantification of the level of resistance of a cultivar or the degree of virulence of a nematode population is an integral part of nematode management. The information allows the breeder to screen for potential resistance genes and to assess the success of introgression of a gene into a genotype. Equally, the information is important to the growers in their choice of PCN management programme. The procedure to determine the resistance of a cultivar or virulence of a population should be efficient, consistent and reproducible (Blok et al., 2018). A protocol for assessing the resistance of a potato genotype to PCN following the European and Mediterranean Plant Protection Organization (EPPO) standards has been well described (Anonymous, 2017a). In this case, assessment is done in a standard one-litre pot in a controlled environment. The test cultivar is inoculated with a defined initial population density of a reference population. The degree of resistance is determined by expressing the reproduction of the nematodes on the test cultivar as a percentage of reproduction on the susceptible control and the level of resistance given in a scale of 1 to 9, with nine representing the highest level of resistance while one represents the highest level of susceptibility (Anonymous, 2017a).

Breeding for resistance to PCN involves screening thousands of potential candidates to identify the desired traits (Gopal, 2015; Mori et al., 2015). Due to the bulkiness of the materials, the use of the EPPO protocol (Anonymous et al., 2017a) is often unfeasible. In classical screening, micro-tubers are commonly used (Dale & de Scurrah, 1998; Anonymous, 2006). These are generated from *in vitro* plantlets and multiplied to produce testing materials (Phillips, 1981 & Gopal, 2015). After planting in pots a reference nematode population is used for inoculation (Anonymous, 2006). Although this method is easy to implement, it is resource and labour intensive (Phillips, 1981) and consequently delays the breeding process. Other resistance screening methods include bulky sowing of potato seedlings (Phillips, 1981), *in vitro* screening in artificial media (Mugniéry *et al*., 1989; Arntzen *et al*., 1994; Fournet *et al*., 2016) and generating testing materials from stem cuttings (Arntzen & Eeuwijk, 1992).

Screening of *in vitro* plants in petri dishes using water agar is also feasible (Mugniéry, 1989), but when handling thousands of genotypes there are high chances of contamination (Mwangi et al., 2019). However, ability to screen breeding materials at the early stages in the breeding programme would highly enhance the breeding process (Plaisted et al., 1984). This may involve screening tissue culture (TC) clones in glasshouse experiments. TC micro-propagation technique allows for

the production of genetically identical clones from an identified plant of interest (Murashige, 1974). Such clones are uniform in size and free of seed-borne diseases making them more suitable for assessing PCN resistance.

Alternatively, the advancements in molecular technology allow for the identification of the genomic region containing resistance genes. Such regions provide genetic markers that are used to screen potential materials (Blok et al., 2018). For example, marker assisted selection (MAS) is used to identify genotypes carrying target resistance genes (Milczarek *et al*., 2011) making it possible to screen a high number of individuals within a short time period. Several genetic markers for the major resistance genes have been identified (Milczarek *et al*., 2011). However, developing DNA-based markers takes time and involves phenotyping the progenies to validate the results (Blok et al., 2018).

1.5 Use of Resistant Cultivars for Integrated PCN Management

Use of resistant cultivars is an effective method of disease management (Rodríguez-Kábana & Canullo, 1992; Starr et al., 2013; Milczarek et al., 2014). Such cultivars are preferred in control of PCN as opposed to application of chemicals. Resistant cultivars restrict the multiplication of nematodes and reduce crop damage thus increasing potato production (Blok et al., 2018). Cultivars with *R* genes offer absolute resistance to specific pathotypes of PCN (Bakker et al., 2006). Although such cultivars are few, in particular for *G. pallida* populations, the utilization of the available cultivars should be integrated with other pest management methods to improve the efficiency and prolong the efficacy (Trudgill., 2014). An integrated approach of nematode management is highly relevant in Kenya because potato production is very intensive and the climate conditions are favourable for PCN (Janssens et al., 2013). Therefore, nematode densities are likely to increase to damaging levels within a short time period. It is possible to integrate the use of resistant cultivars with other nematode control methods such as crop rotation, trap crop and bio-control (López-Lima et al., 2013; Trudgill et al., 2014) to achieve higher impact.

Crop rotation is one of the oldest methods of crop pest and disease management (Rodríguez-Kábana & Canullo, 1992), including for potato cyst nematodes (Franco et al., 1999). A proper crop rotation system is able to reduce the PCN level in the soil and to maintain good soil health (Franco et al., 1999). However, according to Trudgill et al. (2014), the effectiveness of rotation is enhanced when other control measures are included. In this case, the use of resistant cultivars, *R* genes and

QTLs, as well as non-host crops is encouraged. Resistant cultivars allow hatching of eggs and penetration of juveniles, but the nematodes are not able to reproduce. Non-host crops suppress nematode densities in the soil by triggering spontaneous hatching leading to the death of the juveniles due to the absence of a suitable host (Rice, 1985; Devine et al., 1999).

Trap crops fit well into an integrated management system (Scholte, 2000a). The crops stimulating nematode hatch by their root exudates (Franco et al., 1999) allow juveniles to invade the roots, but are not allowed to complete their life cycle (Back et al., 2018). Many trap crops have been identified (Franco et al., 1999; Scholte, 2000b; Dias *et al*., 2012). A good example is *Solanum sisymbriifolium* whose effectiveness in reducing PCN in the field has been intensively studied (Dias *et al*., 2012). In addition, non-tuber bearing *Solanum* spp. are able to induce hatching of nematode eggs in the soil, but some of them are resistant to PCN (Scholte, 2000b). Such trap crops, if well utilized, can supplement the use of resistant potato cultivars. However, use of tuber bearing *Solanum* spp. as trap crop can lead to an increase in nematode populations following untimely destruction of the crop (Scholte, 2000a). In addition, some tubers survive in the soil and emerge as volunteer crop in the subsequent season leading to further nematode reproduction (Scholte, 2000a).

Biological control involves use of predatory nematodes, fungi and bacteria and other organisms in the management of plant parasitic nematodes. Siddiqui & Mahmood (1996) reviewed various fungi used as bio-control agents in the management of parasitic nematodes. A key species among them is *Arthrobotrys oligospora* that produces structures that trap and kill nematode juveniles in the soil (Den Belder & Jansen, 1994). Others like *Paecilomyces lilacinus* (Esser & El-Gholl, 1993), *Pochonia chlamydosporia* (Manzanilla-López et al., 2013) and *Cylindrocarpon destructans* (Morgan-Jones & Rodríguez-Kábana, 1986) parasitize nematode eggs. Bacteria such as *Bacillus subtilis*, *Agrobacterium radiobacter* and several members of *Pseudomonas* spp. have also been used for the biological control of nematodes (Tian et al., 2007). Like other nematode control methods, biological control is more effective when integrated with other management methods (López-Lima et al., 2013).

1.6 Challenges of Resistance Based Management of PCN in Kenya

Most of the chemicals used in the management of nematodes in the past are no more available due to their adverse effects to the environment and human health (Sheridan et al., 2004). Therefore,

potato growers in Kenya and other parts of the world have very few available PCN management options. Key among them is the use of resistant potato cultivars (Molinari, 2011). Such cultivars are effective in their action, easier to use and friendly to the environment (Starr et al., 2013; Milczarek et al., 2014; Davies & Elling, 2015). However, there are several challenges facing the use of resistant potato cultivars in nematode management:

1.6.1 New Introductions and Adaptation

Despite *G. rostochiensis* and *G. pallida* being under strict quarantine regulations worldwide (Anonymous, 2017a), the two species have continued to spread (CABI, 2019). In 2015, *G. rostochiensis* was reported in Kenya (Mwangi et al., 2015) and three years later *G. pallida* was reported (Mburu et al., 2018). Furthermore, in 2019, *G. rostochiensis* was reported in another East African Country - Rwanda (Niragire et al., 2019). Management of newly introduced populations of PCN using resistant potato cultivars can be challenging. This is because there is little information regarding the populations. Resistance based management is species or population specific (Mulder & Van Der Wal, 1997) and therefore adequate knowledge regarding the genetic diversity, population structure, the pathotypes and the biology of the populations is required before resistant cultivars can be deployed. Secondly, PCN are highly adaptable to different environmental conditions. This may present new challenges, for instance with favourable climatic conditions, the Kenyan population may adapt to a non-diapause life style. Equally, farming practices differ with the region and country. For example, potato production in Kenya is done throughout the year with minimum crop rotation (Janssens et al., 2013; Muthoni et al., 2013). All these dynamics need to be taken into account for effective utilization of resistant cultivars.

1.6.2 Virulence Selection

Long-term use of resistant cultivars in nematode management imposes selection pressure on the target population leading to emergence of virulent individuals or shifts in nematode species in favour of another species (Turner, 1990; Young, 1992; Turner & Fleming, 2002). There are reports of virulent PCN populations selected in the field (Mulder & Van Der Wal, 1997; Niere et al., 2014). Similarly, nematode species shifted in the UK due to continued use of cultivars resistant to *G. rostochiensis* (Minnis et al., 2002). *G. rostochiensis* was reduced by the use of *H1* resistance, but *G. pallida* which is more challenging to control with resistant cultivars, proliferated (Minnis et al., 2002; Hockland et al., 2012). According to Eoche-Bosy et al. (2017), *G. pallida* is able to

adapt to QTLs after only a few generations on similar cultivars. This has been observed in laboratory rearing of PCN (Turner et al., 1983; Castagnone-Sereno et al., 2007; Fournet et al., 2013; Beniers et al., 2019). Such selected populations often have higher virulence and fitness (Beniers et al., 1995; Fournet et al., 2016). Loss of resistance efficacy proceeds faster in the presence of mixed nematode species (Spitters & Ward, 1988). Since the two PCN species are present in Kenya and in some cases as mixed populations, deployment of resistant potato cultivars to manage PCN in Kenya should be approached with caution. Since there are no cultivars with full resistance to *G. pallida*, use of cultivars with resistance to *G. rostochiensis* will allow the increase of the latter.

6.2.3 Lack of Adequate Resistant Genotypes

Effective use of resistance-based management of PCN requires the availability of adequate genotypes carrying *R* genes to major nematode populations. So far, it is possible to manage *G. rostochiensis* populations with the available *R* genes (Bakker et al., 2006; Hockland et al., 2012; Minnis et al., 2002). However, there are no cultivars with complete resistance to most populations of *G. pallida*. The only available *R* gene, *H2* gene, is effective against the Pa1 *G. pallida* pathotype (Dalamu et al., 2012) while there are no cultivars with full resistance to pathotype 2/3 of *G. pallida* which is the most prominent in many countries, especially in Europe. Furthermore, there are reports of populations with enhanced virulence following selection on resistant cultivars (Niere et al., 2014).

Globodera rostochiensis and *G. pallida* are present in Kenya (Mwangi et al., 2015; Mburu et al., 2018) yet none of the potato cultivars in the Kenyan potato variety catalogue has resistance to *G. pallida* and only 32% are resistant to some pathotypes of *G. rostochiensis* (Anonymous, 2017b). Most of the listed potato cultivars have not been assessed for resistance to potato nematodes.

1.7 Thesis Outline

This research study was undertaken to address the challenges expounded above. The main objective of the study was to investigate ways of enhancing the use of resistant potato cultivars in the management of potato cyst nematodes in Kenya. As outlined above, use of resistant potato cultivar require information regarding the target population(s) and therefore it is important to study the target population before recommending use of resistant cultivars. On the other hand there are

no adequately resistant potato cultivars especially for *G. pallida* populations and breeding for nematode resistance is resource and time consuming (Davies & Elling, 2015) requiring the phenotyping of thousands of individual genotypes to identify resistance genes. A method for screening materials for resistance at the initial stages of breeding would significantly improve the breeding process (Plaisted et al., 1984). Such a method should be reliable, accurate and consistent (Molinari, 2011).

The specific objectives covered in this thesis were:

a. **To study the biology, pathotype(s) and virulence of *Globodera rostochiensis* populations from Kenya**. Management of the newly introduced PCN populations using resistant potato cultivars is difficult due to lack of basic information regarding the populations. This information is important when deploying resistant potato cultivars. In **Chapter 2,** the biology, pathotype(s) and virulence of Kenyan *G. rostochiensis* are reported and discussed. The feasibility of using resistant cultivars in the management of the nematodes is also discussed considering the cropping systems that are practiced in Kenya.
b. **To investigate the genetic diversity and genetic structure of *G. rostochiensis* from Kenya.** Understanding the genetic diversity and structure of a population helps to identify introduction routes and therefore to limit further spread. Moreover, the information is valuable in identifying new sources of resistance for breeding purposes. In **Chapter 3,** various molecular tools were used to investigate the genetic characteristics of Kenyan *G. rostochiensis* populations compared to populations of the same species from other parts of the world.
c. **To characterize life history traits associated with change of virulence in PCN**. Loss of resistance following continued use of resistant potato cultivars is a major challenge to resistance-based management of PCN. New virulent populations selected in the field have been reported (Niere *et al*., 2014). The study of such populations with higher changed virulence is important for developing sustainable management strategies. In particular, to identify life history traits associated with changes in virulence, which help predict change of virulence in a potato cropping systems. A study on a new resistance breaking virulence type of *G. pallida* from Germany is reported and discussed in **Chapter 4**. We assessed various life history traits associated with change in virulence.

d. **To evaluate the suitability of using *in vitro* potato plants in measuring resistance to PCN.** Unavailability of resistant cultivars against some pathotypes of PCN is attributed to the long breeding processes and in particular the time taken in identifying genotypes with suitable resistance. Long screening processes, followed by phenotyping of the progenies to evaluate the success of the breeding, significantly prolong the time taken to release a new resistant cultivar. In **Chapter 5,** a new test system for screening potato genotypes at the early stages of the breeding process, using *in vitro* derived potato plants is presented.

e. In **Chapter six** the results obtained in the preceding chapters are discussed as a whole.

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Chapter 2: Biology, Pathotype and Virulence of *Globodera rostochiensis* Populations from Kenya

2.1 Abstract

The potato cyst nematodes (PCN), *Globodera rostochiensis* (Woll.) and *G. pallida* (stone) are important pests of potato globally. Due to their extensive damage potential and the challenging management, the nematodes are under strict regulations in many countries. Despite the regulations, the pests continue to spread into new areas and countries. In Kenya, *G. rostochiensis* was first reported in 2015 and *G. pallida* was reported three years later, both in Nyandarua County. This paper reports on the biology, pathotypes and virulence of *G. rostochiensis* populations from Kenya based on glasshouse and laboratory studies. The development of *G. rostochiensis* was assessed on roots of the susceptible cultivar 'Désirée' and resistant cv. 'Laura'. The 'HAR1' populations from Kenya and 'Ecosse' from Germany were not able to complete the life cycle in the roots of the resistant potato cultivar. The penetration of the juveniles into the root did not differ ($P < 0.05$) between populations and cultivars. However, in the resistant cultivar, the juveniles developed into male nematodes. A total of 736 cumulative degree-days (DD_6) were required by 'HAR1' to complete the life cycle on the susceptible cultivar whereas 'Ecosse' completed the life cycle within 645 DD_6. The Kenyan populations lacked obligatory diapause and they were able to hatch immediately after maturity. Consequently, three reproduction cycles could be attained in less than a year, with Kenyan populations, without diapause. The populations from Kenya failed to reproduce on cultivars carrying the *H1* resistance gene. This indicates that they belong to the Ro1/4 pathotype group. On selected potato cultivars, the populations were not able to reproduce on ten out of 13 cultivars tested. The ten cultivars carry the *H1* resistance gene. We therefore conclude that the Kenyan populations of *G. rostochiensis* used in this study belonged to the pathotype group Ro1/4 and that they can be effectively managed with potato cultivars carrying the *H1* resistance gene.

Keywords

Diapause, *Globodera rostochiensis*, *G. pallida*, pathotype, resistance genes, virulence, management

2.2 Introduction

Potato, *Solanum tuberosum* L., is an important food crop cultivated all over the world. It ranks third globally in terms of human consumption. In Kenya, potato is the second most important food crop and it has been earmarked by the government of Kenya for improvement in an effort to ensure food sufficiency (Anonymous, 2016). Production of potato is mainly done by small holder farmers owning, on average, less than 2 ha of potato fields (Gildemacher et al., 2011; Janssens et al., 2013). The total area under production was estimated at 192,341 ha in 2017 (FAO, 2019). However, the productivity of < 10 t ha^{-1} is far below the production potential of > 40 t ha^{-1} reported among the leading potato producers (Anonymous, 2008; Janssens et al., 2013; FAO, 2019). Biotic and abiotic stresses are some of the factors curtailing the production, key among them being pests and diseases (Were et al., 2013). Bacterial wilt, caused by *Ralstonia solanacearum,* and late blight, caused by *Phytophthora infestans,* have been identified as the major potato diseases in Kenya (Muthoni et al., 2013). For a long time, plant parasitic nematodes (PPN) have not been regarded as important potato pests in the country. PPN attack a wide range of agricultural crops and consequently constrain an already erratic global food security (Jones et al., 2013). However, they often go undetected since symptoms associated with their infestation resemble those caused by other biotic and abiotic factors (Mulder et al ., 1997). Until 2015, root knot nematodes (RKN) were the only economically important plant nematodes associated with potato in Kenya. In a study on occurrence and distribution of potato diseases, *Meloidogyne* spp. were identified in 38% of soil samples analysed (Were et al., 2013) while potato cyst nematodes (PCN) were not reported in any of the soil samples.

The PCN, *Globodera rostochiensis* (Woll.) and *Globodera pallida* (Stone) rank second after RKN in their economic importance globally (Jones et al., 2013). They are the most damaging nematode pests of potato. Their status as quarantined pests (Hockland et al., 2012; Anonymous, 2017) has great impacts on the world trade with potatoes and other tuber crops (Faggian et al., 2012). PCN originated in the Andean region of South America (Mai, 1977; Grenier, 2010) and spread to countries where potatoes are grown mainly through movement of contaminated potato material, contaminated soil, movement of farm machinery, among other means (Mai, 1977; Brodie, 1993; Goeminne et al., 2011; Banks et al., 2012). Today, PCN are spread all over the world. They have been reported in several countries in Africa including South Africa (Knoetze., 2006), Algeria

(Mezerket et al., 2018), Morocco and Tunisia (Greco, 1993), Rwanda (Niragire et al., 2019), among others (CABI, 2019).

In 2015, *G. rostochiensis* was reported in Nyandarua County of Kenya (Mwangi et al., 2015), the leading county in potato production. A nationwide survey that followed, led to the discovery of more infested areas and, in addition, *G. pallida* was detected in one potato field within the county (Mburu et al., 2018). There is little information regarding these two nematode species and the impact they pose to the potato industry. Potato production in Kenya is mainly through small holder farmers whose production is limited by small acreage and lack of resources for input (Janssens et al., 2013). They intensively grow potatoes for subsistence use and the excess is sold in the local market (Anonymous, 2016). The production is done throughout the year with minimal crop rotation (Muthoni et al., 2013). Most farmers do not have access to certified seed potatoes and therefore they produce their own seed or source them from the neighbourhood (Gildemacher et al., 2011). Such farm practices allow the nematodes to spread and accumulate to damaging levels.

Quick interventions are required to prevent further invasion and increase of nematode densities to unmanageable levels. An integrated management approach includes proper crop rotation cycles using non-host crops (Evans, 1993), use of trap crops (Dias et al., 2012), application of biological control products (Davis et al., 2018) and use of resistant potato cultivars (Molinari, 2012), among others. Chemical application as a management option is highly restricted due to the adverse effects chemicals have on humans and the environment (Greco, 1993; Sheridan et al., 2004; Faggian et al., 2012). Most nematicides are no longer on the market, leaving farmers with limited options.

Use of the resistant potato cultivars in nematode management is easy for farmers to apply and safe to the environment (Molinari, 2012; Davies & Elling, 2015). Cultivars with complete resistance to *G. rostochiensis* are available to the growers. These cultivars contain major resistance (*R*) genes such as *GroVI* and *H1*, which confer resistance to *G. rostochiensis* (Bakker et al., 2006; Dalamu et al., 2012). *GroVI* is derived from *Solanum vernei* (Jacobs et al., 1996) while the *H1* gene is derived from *Solanum tuberosum* spp. *andigena* (Toxopeus & Huilsman, 1953) and confers resistance to pathotypes Ro1 and Ro4 of *G. rostochiensis* (Turner & Stone, 1984). These *R* genes have been introgressed into many commercial cultivars (Bakker et al., 2006; Dalamu et al., 2012). They are highly effective and have remained stable for decades (Bakker et al., 2003). However, successful use of resistant potato cultivars for the management of PCN requires knowledge about

the characteristics of the target populations (Greco, 1993). Consequently, to utilize resistance in the management of PCN, information regarding the population(s) is desirable. This includes information on the biology, the pathotype(s) and the virulence of the populations on the available resistance genes.

The specific objectives of this study were to: (i) study the development of the 'HAR1' population from Kenya in the roots of resistant and susceptible potato cultivars and compare the development with that of the European population 'Ecosse' (Anonymous, 2006). (ii) Investigate the presence of obligatory diapause in the Kenyan populations. (iii) Determine the pathotype(s) of *G. rostochiensis* populations from Kenya and (iv) assess the virulence of Kenyan populations on selected potato cultivars in comparison to the reference population, 'Ecosse'.

2.3 Materials and Methods

2.3.1 Nematode Populations

Seven populations of *Globodera rostochiensis* were used in this study. Six of the populations were from Kenya and one population was from Germany. Five populations, 'HAR1', 'HAR2', 'RIR', 'KIN1' and KIN2, were sampled in potato fields in Nyandarua County while the sixth population ('TGN') was from Kiambu County. The population 'Ecosse' was obtained from the nematode laboratory JKI-Braunschweig. 'Ecosse' is the official reference population for testing potato genotypes for resistance to *G. rostochiensis* pathotype Ro1 (Anonymous, 2006). Kenyan populations were extracted from sun dried soil samples using a Fenwick can (Fenwick, 1940). Cysts were handpicked and shipped to JKI-Braunschweig where this study was conducted, under strict quarantine conditions. The populations were multiplied twice on the susceptible potato cv. 'Désirée' prior to the study. Except for the cysts used in experiments testing the presence of diapause, the rest of cysts were stored at 4 °C for at least 6 months before they were used in this study to break the diapause.

2.3.2 Planting Material

Potatoes derived from tubers and *in vitro* propagation were used in the study. The tuber derived cultivars were; 'Désirée', 'Laura', 'Albatros', 'Amado', 'Seresta', 'Papageno' 'Belana' and 'Ribera' while 'Connect', 'Caruso', 'Désirée', 'Amanda', 'Performer' and 'Rossini' were *in vitro* propagated. In addition, six differential potato clones (Kort et al., 1977) were used to test the

pathotype of the Kenyan population (Table 2.1). Potato tubers were pre-germinated ahead of the experiments. *In vitro* plants were approx. 3 weeks old and 30 mm tall.

Growth Substrate and Glasshouse Conditions

Experiments were done in loess soil (Müller & Rumpenhorst, 2000; Mwangi et al., 2019). The soil was fertilized at the beginning of the experiment by mixing it with slow release fertiliser ((Osmocote Exact Standard®) 15% N, 9% P2O5, 12% K2O and 2% MgO) at a rate of 1.5 g (kg soil) $^{-1}$. The glasshouse temperature was set at 18 ± 2 °C and relative humidity maintained at a range of 50-70% throughout the experiments. Soil and air temperatures were recorded hourly using Testo® 175T3 temperature loggers. Apart from the experiment on the study of nematode biology, all other experiments were stopped after attaining 1,000 DD_6 at approx. 12 weeks after inoculation. Supplementary light was provided during winter to ensure a minimum of 12 hours light per day. Every experiment was repeated under similar conditions.

Preparation of Inoculum

Plants were inoculated either with full cysts concealed in a nylon mesh or with eggs and juveniles. To prepare the inoculum, a known number of cysts were soaked in tap water overnight and then crushed to free eggs and juveniles (Seinhorst & Den-ouden, 1966). A suspension of eggs and juveniles was prepared and the number adjusted to approx. 500 eggs and juveniles per ml^{-1} water. During inoculation, two, 30 mm deep holes were made in the soil per pot using a plastic rod and nematodes dispensed equally into the holes using a pipette to achieve the desired P_i of five eggs and juveniles ml^{-1} soil.

Sample Processing

Upon termination of the experiments, cysts were extracted from the entire soil per pot by washing the loess through a 250 µl sieve (Mwangi et al., 2019). The cysts containing plant debris were collected on a filter paper (Macherey-Nagel GmBh & 161Co. KG) and left to dry at room temperature for a period of two weeks. Cysts were then separated from the debris using the acetone extraction method (Seinhorst, 1974). Recovered cysts were counted and then crushed (Seinhorst & Den-Ouden, 1966) to estimate the final number of eggs and juveniles per treatment (Mwangi et al., 2019).

2.3.4 Study of Nematode Biology

The development of the 'HAR1' *G. rostochiensis* population in the roots of the resistant potato cv. 'Laura' and the susceptible cv. 'Désirée' was assessed in the glasshouse and compared with the reference population, 'Ecosse'. Eye-plugs were scooped from the pre-germinated tubers of the two cultivars and planted in 192 ml pots. For each of the cultivar-population combinations, fifty eye-plugs were planted. Inoculation was done during planting with ten cysts per pot packed in retrievable bags made up of a nylon mesh and placed next to the eye-plug. Pots were randomized in the support boxes and left on the glasshouse benches and plants were watered as required throughout the experiment.

To assess various developmental stages of the nematodes in the potato roots, four plants per treatment were randomly selected at every sampling date. Sampling was done at 14, 18, 23, 28, 35, 42, 49, 56, 63 and 70 days after planting. Soil adhering to the roots was collected into centrifugation tubes and second stage juveniles (J2s), males and females extracted using the centrifugation flotation method (Anonymous, 2013). Roots were then washed and stained in acid fuchsin (Byrd et al., 1983). The stained roots were cut into approx. 10 mm long sections and macerated in water using an IKA ULTRATURRAX® homogenizer (IKA®-Werke GmbH & Co. KG) at 1 x 10^5 rpm for approx. 30 sec. By this, nematodes at different developmental stages were freed from the root tissues into the water to suspension. The developmental stages of the nematodes were counted from the entire suspension using a 15 ml counting dish under a Nikon® SMZ1270 stereo microscope. Twelve weeks after planting, the experiment was stopped and the final dry cysts extracted from four pots per treatment as described earlier.

2.3.5 Investigating the Presence of Diapause

The presence of diapause in Kenyan populations was investigated on six *G. rostochiensis* populations in two ways. First, the populations were reproduced twice on the susceptible potato cultivar 'Désirée'. The freshly developed cysts from the second reproduction were used in this experiment without storage to break diapause (to break diapause cysts are kept at 4 °C for a minimum of three months). Ten fresh cysts (1^{st} generation cysts), for each of the six populations, were packed in small nylon mesh bags with five replications per treatment and buried in the pot next to the tuber of the susceptible cultivar 'Désirée'. Pots were filled with soil, randomized in the glasshouse, and watered as required. After twelve weeks, the experiment was terminated and cysts

extracted and enumerated as described above. The newly extracted were packed and a new experiment set as explained above, without breaking diapause. The process was repeated as above until four generations were completed.

In a second experiment, the population 'HAR1' was used to test the ability of the newly formed cysts to hatch without diapause. Hatching tests were done in potato root diffusate (PRD) to induce hatching and tap water was used as the control. The PRD was obtained as described by Rawsthorne and Brodie (1986). Twenty newly formed cysts (1st generation cysts) were placed in hatching tubes with four replications per treatment and hatching media (PRD or water) added. The experiment was left in the dark at room temperature for 10 weeks. Hatched juveniles were counted and hatching media replaced with fresh weekly for a period of ten weeks. At the end of the experiment, the cysts were crushed to determine the number of the unhatched eggs. The hatching tests were repeated using the 2nd and 3rd generation cysts without diapause.

2.3.6 Testing the Pathotype

Due to limited availability of differential clones, only the 'HAR1' population was tested twice in glasshouse experiments to test the pathotype of this population on six differential clones (Table 2.1). Pre-germinated tubers of the potato clones were planted into 1000 ml pots with five replications each. Fourteen days after planting, each pot was inoculated with eggs and juveniles of 'HAR1' population to achieve a P_i of five eggs and J2s ml^{-1} soil. As control, plants were inoculated with the Ro1 reference population 'Ecosse'. The pots were left in the glasshouse for twelve weeks and the reproduction assessed at the end of the experiment.

Table 2.1 Differential clones for identification and classification of *G. rostochiensis* pathotypes according to Kort *et al*. (1977).

	Reaction to Pathotypes*				
Cultivar/clone	**Ro1**	**Ro2**	**Ro3**	**Ro4**	**Ro5**
S. tuberosum ssp. *tuberosum* 'Désirée'	+	+	+	+	+
S. tuberosum ssp. *andigena* (*H1*)'Laura'	-	+	+	-	+
S. vernei $(VT^n)^2$ 62.33.3	-	-	-	-	±
S. kurtzianum KTT/60.21.19	-	-	±	±	±
S. vernei GLKS.58.1642.4	-	-	-	+	+
S. vernei 65. 346.19	-	-	-	-	-

* "+" = susceptible ($P_f/P_i > 1$); "-" = resistant ($P_f/P_i < 1$), "±" = partial or uncertain

2.3.7 Virulence Assessment

Two experiments were done to assess the virulence of Kenyan *G. rostochiensis* populations. In the first experiment, the virulence of three *G. rostochiensis* populations ('HAR2', 'KIN1' and 'TGN') from Kenya and the European population 'Ecosse' was assessed on six *in vitro* derived potato cultivars. Plantlets of 'Désirée', 'Amanda', 'Performer', 'Caruso' and 'Connect' and 'Rossini' were removed from the growing media and the roots washed in running water followed by planting into 192 ml pots with five replications each. The pots were randomized in metal boxes and placed on the glasshouse bench. Due to the lack of TC plants, cv. 'Laura' was tested using eye-plugs in 192 ml pots. Fourteen days after planting, each plant was inoculated with eggs and J2s of each of the four nematode populations to achieve an estimated P_i of five eggs and J2s ml^{-1} soil.

In the second experiment, the virulence of the 'HAR1' population from Kenya was tested on seven potato cultivars. The reference population 'Ecosse' was also used for comparison. Tubers of the varieties 'Désirée', 'Albatros', 'Seresta', 'Papageno' 'Belana', 'Ribera' and 'Amado' were planted into 1000 ml pots with 5 replications. Fourteen days after planting, each pot was inoculated with eggs and juveniles to achieve the P_i of five eggs and juveniles ml^{-1} soil. Twelve weeks after inoculation, the experiment was terminated and cysts extracted. Cysts were then enumerated and crushed to estimate the final nematode population density (P_f).

2.3.8 Data Analysis

The cumulative degree-days (DD) were determined by calculating the average daily temperature, minus the base temperature of 6 °C (Mugniéry, 1978). The final nematode density (P_f) was determined by multiplying the number of cysts per pot by the mean number of eggs and juveniles per cyst. The reproduction factor (R_f) was calculated by dividing the final nematode density (P_f) by the initial population (P_i, $R_f = P_f/P_i$). The relative susceptibility (R_s) in percentage of a cultivar was determined using the formula $R_s = P_{f\ \text{test cultivar}}/P_{f\ \text{susceptible control cultivar}}$ x 100, where susceptible cv. 'Désirée' was used as the reference cultivar. The resistance level was then rated using the standard scoring notation where R_s < 1% = 9; 1.1-3% = 8; 3.1-5% = 7; 5.1-10% = 6; 10.1-15% = 5; 15.1-25% = 4; 25.1-50% = 3; 50.1-100% = 2; > 100% = 1. Where 1 is the most susceptible and 9 the most resistant (Anonymous, 2006). Mean numbers of nematodes and cysts recovered in each of the experiments were tested for normality and homogeneity of variance using the Shapiro test and Levene's test, respectively. There was no significance difference ($P < 0.05$)

between the repeated experiments testing diapause and testing virulence with tubers therefore the data were pooled prior to the analysis of variance. Data generated from the study of biology and presence of diapause were analysed using ANOVA. Means that were significantly different ($P \leq 0.05$) were separated using Tukey's HSD test. Kruskal-Wallis test was used to analyse data on virulence assessed on *in vitro* potato plants. Treatments that were significantly different ($P \leq 0.05$) were separated using Kruskal Post Hoc test. Statistical analysis was done with statistical software R (version 3.6.0).

2.4.0 Results

2.4.1 Study of Nematode Biology

The results of the two repeated experiments were similar. In the first experiment, the J2s were extracted from the roots in all treatments 14 days after inoculation (DAI), at 171 DD_6. The mean number of J2s recorded did not differ ($P > 0.05$) among cultivars and populations (Fig. 2.1). Besides, few third stage juveniles (J3s) were also detected in cv. 'Désirée' treatment with 'Ecosse' population. 'HAR1' J3s were detected in the roots of cv. 'Désirée' 18 DAI at 237 DD_6. At this time, the number of J3s was higher ($P < 0.05$) in cv. 'Désirée' treatment with 'Ecosse' compared to the same cultivar inoculated with 'HAR1'. Also, the number of J2s in the roots varied among treatments ($P < 0.05$) with higher numbers being recorded on cv. 'Désirée' and cv. 'Laura' by 'Ecosse' compared to 'HAR1' in cv. 'Désirée' and cv. 'Laura'. The first J3s were detected in the roots of the resistant cultivar 23 DAI at 302 DD_6. Overall, the number of J3s was higher in the susceptible cultivar with 'Ecosse' compared to the rest of the treatments. In addition, fourth stage juveniles (J4s) were detected in all treatments except cv. 'Laura' with 'HAR1' where J4s appeared 28 DAI. Twenty eight days and 367 DD_6 were required for the first males and females to emerge from 'Désirée'. Females were present in significantly higher numbers ($P < 0.05$) in 'Désirée' inoculated with 'Ecosse' than with 'HAR1'. From 35 DAI, males were detected in the roots of cv. 'Laura', for both populations, until 63 DAI when the numbers diminished (Fig. 2.1). The number of females extracted from the roots increased steadily from 35 DAI with more 'Ecosse' females counted on the susceptible cultivar compared to 'HAR1' throughout the experiment. However, no females were detected in resistant cv. 'Laura' for both populations. The first brown 'Ecosse' cyst was isolated from the soil 49 DAI at

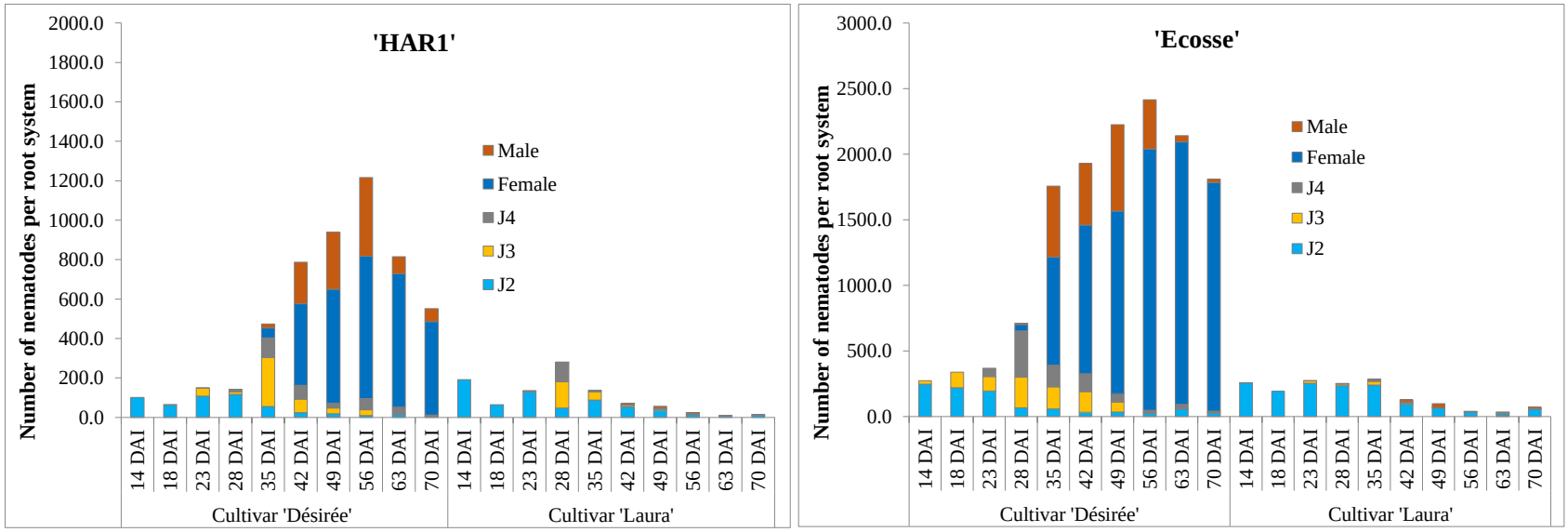

Figure 2.1 Experiment 1 showing the mean number of different developmental stages of *Globodera rostochiensis*, 'HAR1' and 'Ecosse' population in resistant cv. 'Laura' and susceptible cv. 'Désirée' in the glasshouse.

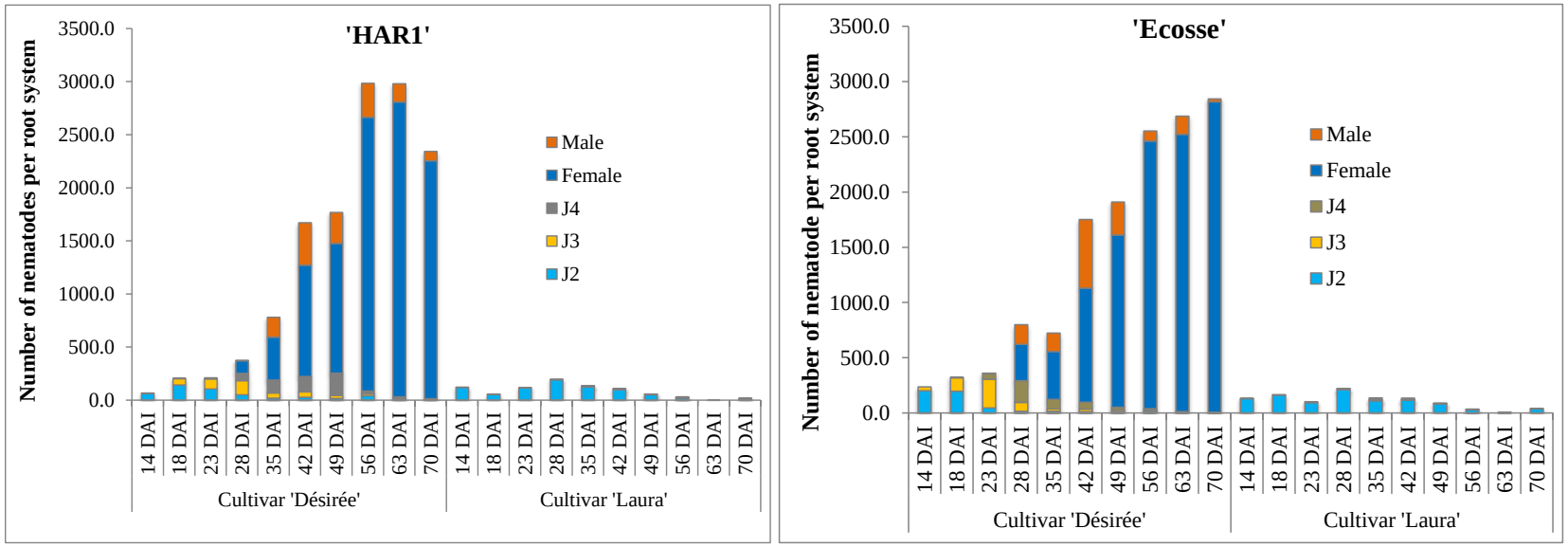

Figure 2.2 Experiment 2 showing the mean number of different developmental stages of *Globodera rostochiensis*, of 'HAR1' and 'Ecosse' population in resistant cv. 'Laura' and susceptible cv. 'Désirée' in the glasshouse.

646 DD_6 and for 'HAR1' 7 days later at 741 DD_6 (Fig. 2.1). The mean numbers of cysts at the end of the experiment were 918 and 705 for 'Ecosse' and 'HAR1', respectively.

The number of developing 'HAR1' and 'Ecosse' nematodes recorded in the roots and soil in the repeat experiment were similar in all treatments. However, some developmental stages were detected earlier than in the first experiment. For instance, at 14 DAI, both J2s and J3s were detected in the roots in all treatments. At this date, the cumulative degree-days were slightly higher (182 DD_6) than in the first experiment (Fig. 2.2). Similarly, J4 were detected 5 days earlier in experiment two (18 DAI at 239 DD_6). The first 'Ecosse' females were detected in 'Désirée' on 23 DAI at 309 DD_6, this was 5 days earlier than in the first experiment while 'HAR1' females were detected 5 days later (28 DAI at 382 DD_6). Only males were detected in the roots of the resistant cv. 'Laura' and in low numbers. The number of days taken to complete the life cycle was similar to the first experiment with cumulated DD_6 of 645 and 736 DD_6 for 'Ecosse' and 'HAR1', respectively. The average number of cysts per root system at the end of the experiment was 956 and 876 for 'Ecosse' and 'HAR1', respectively.

2.4.2 Investigating the Presence of Diapause

All populations tested were able to reproduce without diapause (Fig 2.3). The R_f values varied significantly among generations for 'RIR' and 'KIN1', but not the other four populations. In the two former populations, reproduction of the 2nd second generation was higher ($P < 0.05$) than the 3rd generation. The overall reproduction in the 2nd generation ranged from $R_f = 9.5 \pm 2$ in 'TGN' to $R_f = 26.5 \pm 3$ in 'RIR' (Fig 2.3). In the 4th generation, the lowest R_f was recorded for 'HAR1' ($R_f = 6.4 \pm 1$) while 'KIN1' had the highest ($R_f = 19.3 \pm 4$).

When incubated in PRD for a period of 10 weeks up to 68% of the 1st generation cysts hatched, compared to 14% hatch in water. The hatching rate was lower in the subsequent generations with 55% and 44%, respectively (Fig. 2.4). The hatching in water was less than 2% in the third and the 4th generation cysts.

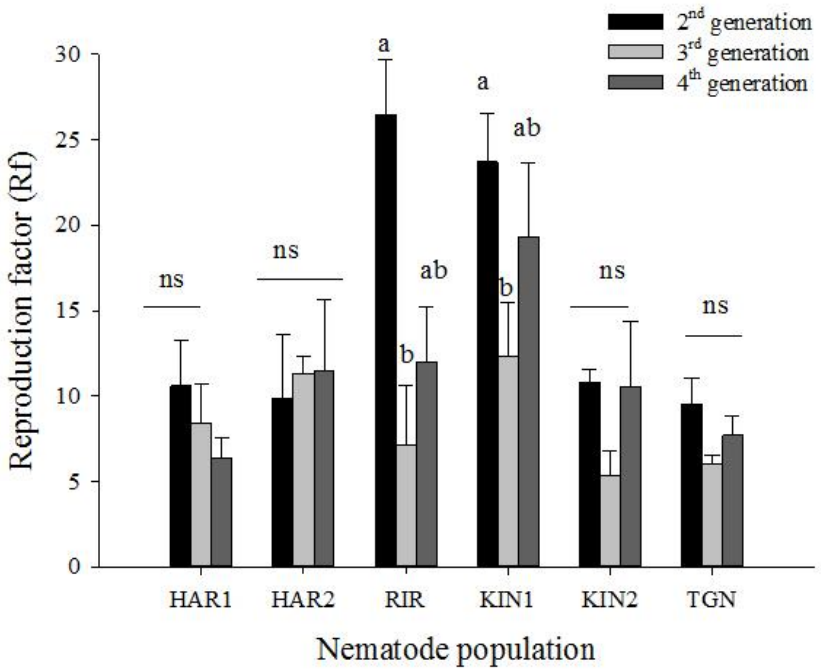

Figure 2.3 Mean reproduction factor (± SE) of six populations of *Globodera rostochiensis* from Kenya on the susceptible cultivar 'Désirée' for three successive generations without diapause. Vertical bars show the standard error of the mean (n = 6). Means within a population with the same small letter are not significantly different (P > 0.05).

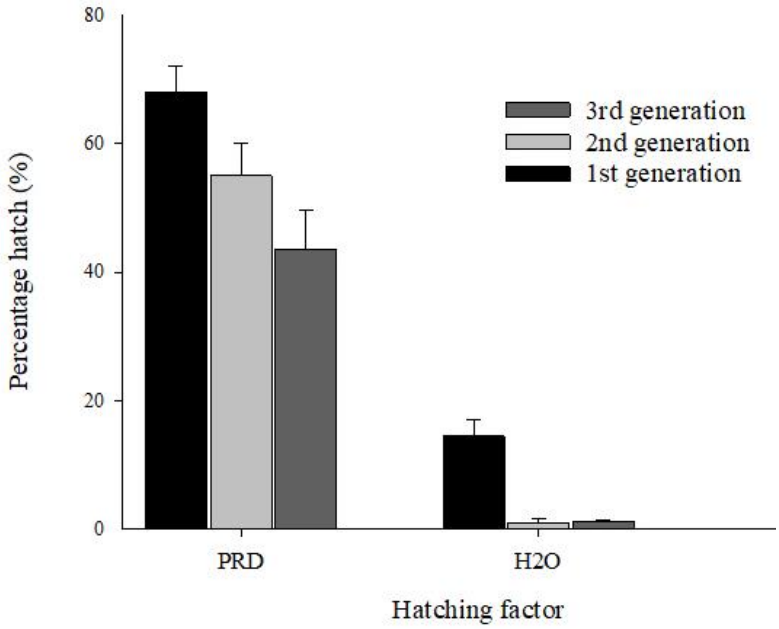

Figure 2.4 Percentage hatch of *Globodera rostochiensis* 'HAR1' population in PRD for three successive generations of cysts without diapause. Vertical bars are mean percentage ± standard error (n = 4).

2.4.3 Tests for Pathotype

The 'HAR1' and 'Ecosse' populations had a high R_f/P_i ratio on susceptible cv. 'Désirée' with R_f/P_i of 61.4 and 46.6, respectively (Table 2.2). There was no cyst produced on *S. tuberosum* cv. 'Laura' and *S. vernei,* 65.346.19 ($P_f/P_i = 0$), the two clones were therefore resistant to the two populations. The P_f/P_i value on *S. vernei,* 58.1642.4 was < 1 for both populations. Equally, both populations had a low P_f/P_i on *S. vernei*, 62.33.3 where 'HAR1' had a P_f/P_i of 3.2 compared to 'Ecosse' with 2.4. Only 'HAR1' had a P_f/P_i ratio of more than one ($P_f/P_i = 1.1$) on *S. kurtzianum,* 60.21.19 (Table 2.2).

Table 2.2 P_i/P_f ratios of *Globodera rostochiensis* populations 'HAR1' and 'Ecosse' on six differential potato clones (Kort et al., 1977).

		Population		Status*	
Potato clones	**Resistance**	**'HAR1'**	**'Ecosse'**	**'HAR1'**	**'Ecosse'**
S. tuberosum cv. 'Désirée'	None	61.4	46.6	+	+
S. tuberosum cv. 'Laura' (*H1*)	Ro1,4	0.0	0.0	-	-
S. kurtzianum, 60.21.19	Ro1,2	1.1	0.4	+	-
S. vernei, 58.1642.4	Ro1,2,3	0.1	0.1	-	-
S. vernei, 62.33 .3	Ro1,2,3,4	3.2	2.4	+	+
S. vernei, 65.346.19	Ro1,2,3,4,5	0.0	0.0	-	-

* (+) indicates a multiplication rate (P_f/P_i) >1.0 (susceptible), and (-) indicates a P_f/P_i <1.0 (resistant)

2.4.4 Virulence Assessment

The reproduction factor varied between the populations 'Ecosse', 'HAR2', 'KIN1' and 'TGN'. In addition, the populations had a higher R_f in experiment one compared to experiment two (Table 2.3).

Reproduction of the four populations on *in vitro* cultivar 'Désirée' ranged from an R_f of 14.75 to 37.27 in experiment one compared to experiment two ($3.53 < R_f < 19.92$). There was no significant difference in R_f of the populations, on the reference cv. 'Désirée', except for 'HAR2' in experiment one and 'KIN1' in experiment two, which had significantly lower R_f ($P < 0.05$). All populations had significantly lower reproduction ($P < 0.05$) on the four potato cultivars 'Rossini', 'Caruso', 'Amanda' and 'Laura' with an $R_f < 1$ compared to cv. 'Désirée', 'Connect' and 'Performer' in both experiments (Table 2.3).

The population 'HAR2' had the lowest reproduction on 'Connect' in the first experiment with R_f = 18.43 compared to the other populations whose R_f ranged from 21.8 to 31.7. In the second

experiment, all populations had a similar R_f on 'Connect' except 'KIN1' (R_f = 2.) that was significantly ($P < 0.05$) lower. On the cultivar 'Performer', populations 'Ecosse', 'HAR1' and 'KIN1' had similar reproduction while 'TGN' had the lowest reproduction (R_f = 1.19) in experiment one. The relative reproduction of all populations on 'Performer' was low ($R_f \leq 1$) in experiment two (Table 2.3).

Table 2.3 Response of seven potato cultivars to four populations of *Globodera rostochiensis* under greenhouse conditions.

Experiment 1				
Cultivar	'Ecosse'	'HAR2'	'KIN1'	'TGN'
'Désirée'	28.43* ± 12.0^{a,A}	14.73 ±9.6^{a,B}	24.97 ± 10.5^{a,A}	37.27 ± 22.7^{a,A}
'Connect'	31.70 ± 9.3^{a,A}	18.43 ± 5.2^{a,B}	30.84 ± 15.3^{a,A}	21.80 ± 9.4^{a,A}
'Performer'	2.37 ± 1.3^{b,A}	1.74 ± 1.3^{bA,B}	2.45 ± 1.9^{b,AB}	1.18 ± 0.9^{b,B}
'Rossini'	0.02 ± 0.1^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.01 ± 0.0^{c}
'Caruso'	0.02 ± 0.1^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.02 ± 0.1^{c}
'Amanda'	0.00 ± 0.0^{c}	0.01 ± 0.0^{c}	0.02 ± 0.1^{c}	0.05 ± 0.1^{c}
'Laura'**	0.00 ±0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}
Experiment 2				
'Désirée'	10.07 ± 1.5^{a,A}	8.11 ± 1.5^{a,A}	3.53 ± 0.3^{a,B}	12.92 ± 0.8^{a,A}
'Connect'	7.47 ± 1.1^{a,A}	13.31 ± 1.9^{a,A}	2.42 ± 0.3^{a,B}	12.25 ± 2.7^{a,A}
'Performer'	0.53 ± 0.1^{b,AB}	0.35 ± 0.1^{b,B}	0.85 ± 0.2^{b,A}	1.00 ± 0.3^{b,A}
'Rossini'	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.04 ± 0.1^{c}	0.01 ± 0.0^{c}
'Caruso'	0.00 ± 0.0^{c}	0.08 ± 0.3^{c}	0.00 ± 0.0^{c}	0.02 ± 0.1^{c}
'Amanda'	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.05 ± 0.0^{c}
'Laura'**	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}

*Reproduction factor (R_f) ± SE. Means of R_f within the same column in each experiment followed with similar lowercase letters are not significantly different ($P > 0.05$, $n = 10$). Similarly, means of R_f within the same row in each experiment followed with similar capital letters are not significantly different ($P > 0.05$).
** Reproduction on cv. 'Laura' was tested using tubers due to lack of *in vitro* plants of this cultivar.

Despite the variability in the reproduction of the four populations between the two experiments, the relative susceptibility (R_s) of the cultivars to the nematode populations did not differ (Table 2.4). The four potato cultivars, 'Rossini', 'Caruso', 'Amanda' and 'Laura' had an R_s of less than 1% and therefore were ranked as highly resistant (score = 9) according to Anonymous (2006). The cultivar 'Connect' was ranked as highly susceptible (score = 1-2) to all populations tested with a relative susceptibility of over 76.7% (Table 2.4). The relative susceptibility of the cultivar 'Performer' to the populations tested ranged from 5% to 17%. The cultivar achieved score of 6 for 'Ecosse', 'HAR1' and 'TGN' and a score of 4 for 'KIN1' (Table 2.4).

Table 2.4 Mean relative susceptibility (R_s %) of six potato cultivars to four *G. rostochiensis* populations: 'Ecosse', 'HAR2', 'KIN1' and 'TGN'.

	'Ecosse'		'HAR2'		'KIN1'		'TGN'	
Cultivar	R_s (%)	Score	R_s (%)	Score	R_s (%)	Score	R_s (%)	Score
'Connect'	92.9	2	144.6	1	96.1	2	76.7	2
'Performer'	6.8	6	8.1	6	17.0	4	5.5	6
'Rossini'	0.1	9	0.0	9	0.6	9	0.1	9
'Caruso'	0.1	9	0.5	9	0.0	9	0.2	9
'Amanda'	0.0	9	0.1	9	0.1	9	0.3	9
'Laura'*	0.0	9	0.0	9	0.0	9	0.0	9

The table shows percentage relative susceptibility (R_s) and the resistance ranking of seven potato cultivars. R_s (%)= $Pf_{test\ cultivar}/Pf_{standard\ susceptible\ control\ cultivar} \times 100\%$) and the resistance level (score) determined using the EPPO score scale (1 (>100), 2 (50.1–100%), 3 (25.1–50%), 4 (15.1–25%), 5 (10.1–15%), 6 (5.1–10%), 7 (3.1–5%), 8 (1.1–3%), 9 (≤1%). Where 9 represent the highest level of resistance and 1 represent the least resistant.
* Reproduction on cv. 'Laura' was tested using tubers due to lack of *in vitro* plants of this cultivar.

Reproduction of 'HAR1' and 'Ecosse' was very low on, 'Albatros', 'Belana', 'Ribera', 'Amado', 'Seresta', and 'Papageno' with an $R_f < 1$ and R_s of less than 1% based on experiments (Table 2.5). The six cultivars scored = 9 in resistance. Reproduction of both populations on cv. 'Désirée' was high but not different ($P > 0.05$), although 'HAR1' had higher R_f of 56.6 compared to 'Ecosse' R_f of 51.1(Table 2.5).

Table 2.5 Response of seven potato cultivars to 'HAR1' and 'Ecosse' populations of *Globodera rostochiensis* under glasshouse conditions.

	'HAR1'			**'Ecosse'**		
Cultivars	$\boldsymbol{R_f}$ **± se**	$\boldsymbol{R_S}$ **(%)**	**Score**	$\boldsymbol{R_f}$ **± se**	$\boldsymbol{R_S}$ **(%)**	**Score**
'Désirée'	56.56 ± 8.51*	100	1	51.07 ± 4.37*	100	1
'Albatros'	0.09 ± 0.07	0.16	9	0.02 ± 0.02	0.04	9
'Belana'	0.10 ± 0.05	0.18	9	0.00 ± 0.00	0.00	9
'Ribera'	0.08 ± 0.03	0.14	9	0.00 ± 0.00	0.00	9
'Amado'	0.00 ± 0.00	0.00	9	0.00 ± 0.00	0.00	9
'Seresta'	0.00 ± 0.00	0.00	9	0.00 ± 0.00	0.00	9
'Papageno'	0.01 ± 0.02	0.02	9	0.00 ± 0.00	0.00	9

The table shows mean reproduction factor (R_f) ± SE, relative susceptibility (Rs) and respective resistance score of seven potato cultivars. Means within the same column and raw with similar asterisk (*) are not significantly different ($P > 0.05$, $n = 10$)

2.5 Discussion

Overall, the Kenyan PCN populations studied belonged to the pathotype Ro1/4 and required higher cumulative degree-days compared to the temperate population 'Ecosse'. However, they all did not require diapause to hatch.

The resistant and susceptible potato cultivars 'Laura' and 'Désirée', respectively allowed the hatching and the penetration of J2s in the roots. The number of J2s in the roots 14 DAI was similar despite of the differences in resistance of the two cultivars to *G. rostochiensis*. The overall, higher numbers of nematodes in the plants inoculated with 'Ecosse' compared to those inoculated with 'HAR1' in the first experiment can be explained by the difference in the size of the cysts used for inoculation. 'Ecosse' cysts were bigger in size compared to 'HAR1' cysts that varied in size. Bigger cysts contain more eggs and they are often associated with increased fitness (Fournet et al., 2016). In the repeat experiment, only bigger cysts of the two populations were used, and the number of nematodes of the two populations isolated from the same variety did not differ.

There was no difference in the development of 'HAR1' and 'Ecosse' in the roots of the resistant potato cultivar 'Laura' with no females of 'HAR1' and 'Ecosse' emerging. However, males of both populations were detected in the roots and soil. Failure by the females to complete the life cycle in the roots of a resistant cv. 'Laura' is attributed to the presence of the *H1* resistance gene that confers resistance to pathotypes Ro1 and Ro4 of *G. rostochiensis* (Bakker,et al 2006; Dalamu et al., 2012). This *R* gene restricts the formation of the syncytium, a feeding structure within the host root. The resistance response involves a hypersensitive reaction leading to necrosis and death of cells surrounding the syncytium (Smant et al., 2018). This leaves the developing nematode without enough food and as a result most of them develop into males that do not require a lot of resources (Trudgill, 1991; Moens et al., 2018). The inability of the female to complete the life cycle in the roots of the cultivar carrying *H1* gene strongly suggest that 'HAR1' belongs to the Ro1/4 pathotype group.

The Kenyan 'HAR1' population required more than 736 degree-days (DD_6) to complete its life cycle compared to 'Ecosse' (645 DD_6). The DD_6 days recorded in our study were higher than what has been reported in other studies. For example, *G. rostochiensis* were able to complete the life cycle in 401 DD_6 Ebrahimi et al. (2014). Greco et al. (1988) found that *G. rostochiensis* in the regions characterized by temperate climate required about 168 DD_{10} while those from subtropical

climate needed 450 DD_{10}. In a different study, Philis (1980) found that *G. rostochiensis* needed 529 DD_{10} for the life cycle to be completed. The differences in the DD recorded in these studies resulted from the methods used in the estimation. For example, Philis (1980) calculated the degree-days using the base temperature (minimum heat threshold required by the nematode to develop) of 10 ^{o}C. The calculation was done from the time of root invasion until the time when eggs containing embryos were noticed. Ebrahimi et al. (2014), on the other hand, used 6 °C as the base temperature to calculate DD required to complete the life cycle from the J2s of one generation to the J2s of the next generation. We also used a base temperature of 6 °C but we calculated the DD from the day of inoculation (planting day) to the day when the first cyst was detected in the soil. However, due to high temperature and long sampling intervals (5 to 7 days) there was overlapping of nematode life stages making it difficult to obtain accurate estimates of DD_6 required by the populations to complete their life cycle. The final number of cysts was lower than the number of females recorded in the experiment. This indicates that some females did not form eggs or the host roots died before the females could complete the life cycle. However, the data generated in this study indicate that the two nematode populations are avirulent to on cv. 'Laura' and that the Kenyan population requires more DD_6 than the European population 'Ecosse'.

While temperate PCN populations often undergo a diapause period before they can hatch (Moens et al., 2018) and complete their life cycle, the Kenyan populations lacked obligatory diapause and up to 68% of the eggs were able to hatch immediately after completing their life cycle. This allowed for three successive generations in nine months and the newly developed cysts were able to hatch immediately in PRD. Janssen et al. (1987) described a method of avoiding diapause in PCN. It involved crushing newly formed cysts before they undergo desiccation and inoculating them on a host. In our case, we allowed the cysts to undergo complete desiccation and we did not crush them to expose eggs. The cysts were wrapped in a nylon mesh to distinguish them from newly formed cysts at the end of the experiment. Our approach therefore closely simulated what happens in the field.

The ability of PCN nematodes to complete more than one generation on the same host has been reported by Jimenez-Perez et al. (2009) who recorded a full cycle of the second generation on the same host. They also found that the juveniles hatched throughout the year. In Italy, Greco et al. (1988) monitored the development of the nematodes and found two generations on the same host

during the long potato growing season. A similar trend has been reported by Philis (1980). Our study used a different approach; we did not monitor the presence of second generation on the same host because the plants we used aged quickly and could not support a second generation. Nevertheless, we tried to simulate what happens in Kenya where farmers harvest potatoes and new potato tubers are planted immediately in order to achieve as many crop cycles as possible.

Potato production in Kenya is done twice a year, during the long rainfall season between March and July and during the short rainfall season from October to December (Janssens et al., 2013; Were et al., 2013). In some isolated cases, farmers are able to achieve three potato crops per year under irrigated agriculture. Moreover, farmers practice intensive mono cropping of potato with minimum crop rotation (Muthoni et al., 2013) and they allow the proliferation of volunteer potato crops between two potato crop seasons since volunteer potatoes produce early tubers for family consumption. As PCN population density in the soil increases with increase in the number of generations per year (Greco et al., 1993), this means that the aforementioned farm practices create a highly conducive environment for multiple nematode generations and build-up in the soil thus complicating management.

Both 'HAR1' and 'Ecosse', which is a designated Ro1 (Anonymous, 2006) population, had a very high reproduction (P_f/P_i) on *S. tuberosum* cv. 'Désirée' that is susceptible to all reported PCN pathotypes. They were, however, not able to reproduce on *S. tuberosum* cv. 'Laura', *S. vernei* 65.346.19 and *S. vernei* 58.1642.4 ($P_f/P_i < 1$). The *S. vernei*, 65.346.19 clone is resistant to all *G. rostochiensis* pathotypes, but susceptible to *G. pallida* pathotypes (Kort et al., 1977). Failure by 'HAR1' and 'Ecosse' to reproduce on this clone confirmed that they were pure *G. rostochiensis* populations and not a mixture of two species. Cultivar 'Laura' carries the *H1* gene that confers resistance to Ro1 and Ro4 of *G. rostochiensis* (Bakker et al., 2006). Therefore, is it most likely that the 'HAR1' population belongs to the Ro1/4 pathotype group. Mugniéry et al. (1989) grouped Ro1 and Ro4 together as avirulent pathotypes on the *H1* gene. This was supported by Nijboer & Parlevliet (1990) who found the two pathotypes to have similar virulence patterns. Low reproduction of 'HAR1' on *S. kurtzianum*, 60.21.19 and *S. vernei*, 62.33.3 indicates a degree of heterogeneity within the population.

The pathotyping scheme by Kort et al. (1977) has been criticized particularly for using reproduction rate of >1 or <1 as borderline to separate pathotypes (Mugniéry et al., 1989; Trudgill,

1985; Nijboer & Parlevliet, 1990; da Cunha et al., 2012). This makes it difficult to designate the pathotype in cases when the value is very close to one (Greco et al., 2007). A good example is the reproduction of 'HAR1' on *S. kurtzianum*, 60.21.19 where P_f/P_i was 1.1 ± 0.4. Such a small difference may arise from differences in experimental setup leading to the population being designated as the wrong pathotype. However, there is no alternative system available for classification of *G. rostochiensis* populations and the Kort et al. (1977) scheme continues to provide relevant information that is critical in the management of PCN.

Ten out of thirteen cultivars tested were resistant to Kenyan *G. rostochiensis* populations ($P_f < 1$), but not all the information on the source of resistance of these cultivars is publicly available. The *H1* gene has been introgressed into the cultivars 'Seresta', 'Amado', 'Amanda', 'Caruso', 'Rossini' and 'Laura'. Like HAR1, the Kenyan populations 'KIN1' and 'TGN' were not able to reproduce on cultivars having the *H1* gene and therefore they probably also belonged to the Ro1/4 pathotype group. In addition, the Kenyan populations did not have unusual virulence compared to 'Ecosse' and therefore we concluded that they belong to the same pathotype.

The cultivar 'Performer' exhibited partial resistance to the Kenyan populations. The differences in the reproduction of Kenyan populations on cv. 'Désirée', 'Connect' and 'Performer' between the first and the second experiment might be explained by lack of resistance or the presence of QTLs. Reproduction on such cultivars is strongly influenced by genotype-environment interactions (Trudgill, 1985) and therefore the R_f might differ from one experiment to another.

Information on the pathotype and virulence of a *G. rostochiensis* population is important in guiding breeding and management programmes. The Kenyan populations studied were all pathotype Ro1/4, this means that they can be properly managed using potato cultivars carrying the *H1* gene. Indeed, most of the potatoes tested were resistant to all the populations and can therefore be recommended for use by Kenyan growers. There is a need to test more potato cultivars available in Kenya to establish their level of resistance.

Sustainable management of *G. rostochiensis* using *HI* gene should be in a context of integrated pest management, otherwise persistent use of these cultivars imposes selection pressure on the populations. This was reported in the UK where continued overuse of potatoes resistant to Ro1, which was the dominant pathotype in the UK, led to the selection and increase of *G. pallida*, which

is more difficult to control. *G. pallida* was reported in Kenya (Mburu et al., 2018) at low frequency and care should be taken to prevent the increase and spread of this species. The use of resistant cultivars tested in this study would significantly decrease nematode accumulation resulting from lack of diapause as reported in this study. Pathotype Ro1/4 may be the prevalent pathotype in Kenya; however, there is need to test the pathotypes and virulence of more populations before deploying resistant cultivars.

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Chapter 3: Genetic Diversity and Population Structure of Kenyan Populations of *Globodera rostochiensis*

3.1 Abstract

Recently, the potato cyst nematode, *Globodera rostochiensis,* was detected in potato growing regions of Kenya. Potatoes in Kenya are predominantly grown by smallholder farmers and reinforcing quarantine regulations at the farm level is impractical, whereas at the same time farm practices create a good environment for nematodes to thrive. Effective utilization of resistant cultivars requires knowledge about the genetic composition of the nematode populations. In this study, genetic diversity and population structure of six *G. rostochiensis* populations from Kenya were analysed using 12 microsatellite markers. The populations showed a similar level of genetic diversity. Two populations showed a significant heterozygote deficit, the four other populations were at the Hardy-Weinberg equilibrium. Only one population was genetically differentiated from the others. A factorial correspondence analysis with populations from Europe, South-America and North America showed that Kenyan populations grouped with all those populations, except two populations from Bolivia. However, a structure analysis excluding the Bolivian populations clearly showed that the Kenyan populations formed a distinct genetic structure cluster from the other populations. The analysis could not identify the putative source population. The results provide important information in support of a control programme Kenyan PCN populations.

Keywords

Genetic diversity, Kenya, microsatellite markers, potato cyst nematodes, population structure

3.2 Introduction

Potato Cyst Nematodes (PCN), *Globodera rostochiensis* and *G. pallida* are economically important pathogens of potato. The nematodes feed and complete their life cycle in the roots of the hosts in the Solanaceae plant family such as tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*) among other *Solanum* spp. (Sullivan et al, 2007). PCN are characterized by high reproduction rates and survival capability. They reproduce sexually, eggs are formed in an encapsulated survival structure known as cyst that enables them to survive in the soil for several years (Moens et al., 2018). Due to their highly destructive nature, PCN are under strict quarantine regulations in many countries (Anonymous, 2017).

The origin of the PCN is in the Andean mountains of South America (Mai, 1977; Grenier et al., 2010). The nematodes were introduced into Europe through contaminated breeding materials in the mid-19th century (Turner & Evans, 1998). The pests have since spread to other countries that produce potatoes (CABI, 2019) and Europe is regarded as an introductory route of PCN to the rest of the world (Turner & Evans, 1998). Originally, these nematodes were considered a problem in the temperate regions, but they have spread to tropical and subtropical areas in the last century (CABI, 2019). As poor mobility of the pest cannot account for the dispersal, the key dispersal mechanisms include long distance movement of infested potato tubers, contaminated machinery, foot wares, floods and wind, among others (Brodie, 1993; Goeminne et al., 2011; Banks et al., 2012; Alenda et al., 2014).

Potatoes are the second most important food crop in Kenya after maize (Janssens et al., 2013; Anonymous, 2016a). The production is mainly done in high-altitude areas from 1,500 to 3,000 m above sea level (Janssens et al., 2013) where the temperatures are moderate and the precipitation is sufficient thoughout the year. The production is mainly by small holder farmers with less than 2 ha under potato production. The total area under potato production in the country was estimated at 192,341 ha^{-1} in 2017 (FAO, 2019) with a productivity of less than 10 t ha^{-1} (Janssens et al., 2013).

In Kenya, *G. rostochiensis* was first detected in the highland region of Aberdare, Nyandarua county in 2015 (Mwangi et al., 2015), which is the leading county in potato production. Subsequent surveys in 2017/2018 revealed that the pest was also present in the neighbouring counties and, in addition, the second PCN species, *G. pallida*, was detected in one potato field within the same county (Mburu et al., 2018). It is not yet known where and when the nematodes were introduced into the country. However, a study on the status of seed potatoes in Kenya in early the 1980s did not find any of the PCN species. Indeed, the report highlighted the risks of unregulated importation of seed potatoes to the potato industry (Njoroge, 1982).

PCN are under strict quaratine regulations in Kenya (Njoroge, 1982). Potato farming is characterized by intensive monocroping and only approx. 5.5% of farmers practice crop rotation with crops such as maize or beans (Muthoni et al., 2013). They often use own generated seed potatoes or make use of neighbour's ware potatoes (Muthoni et al., 2013; Kamau et al., 2019). In

some cases, farmers are able to achieve up to three potato crops per year. The good climatic conditions coupled with the aforementioned farm practices create a conducive environment for nematode proliferation and all together impede the implementation and reinforcement of quarantine regulations at the farm level. Deployment of resistant potato cultivars has the potential of reducing nematode populations below the damage threshold (Molinari, 2011).

The utilization of resistant cultivars in the management of PCN requires knowledge regarding the nematode population(s) present. Several *G. rostochiensi* populations from Kenya all belonged to Ro1/4 pathotype but also showed some quantitative variation in virulence (Chapter 2). Information on the genetic variability and the evolutionary potential of nematode populations is essential in order to develop stable strategies for PCN management using resistant cultivars and to reduce the probability of rapid PCN adaptation to the available resistance genes (McDonald & Linde, 2002; Mundt, 2014). Understanding the extent of heterogeneity within the Kenyan *G. rostochiensis* populations and possible introduction pathways can also help to prevent further introduction and spread (Blacket et al., 2019).

Microsatellite markers constitute a valuable tool for investigating cyst nematode genetic diversity and population structure since they are highly polymorphic and suitable for genetic study (Selkoe & Toonen, 2006). This study aimed to i) investigate the genetic diversity and population structure of six potato cyst nematodes populations from Kenya using microsatellite markers, and ii) explore putative sources of introduction using similar data from *G. rostochiensis* populations sampled in Europe, South America and North America (Boucher et al., 2013).

3.3 Materials and Methods

3.3.1 Nematode Populations

The six populations of *Globodera rostochiensis* from Kenya were extracted from soil samples obtained from PCN infested fields in Nyandarua and Kiambu counties with a long history of potato production (Fig. 3.1).

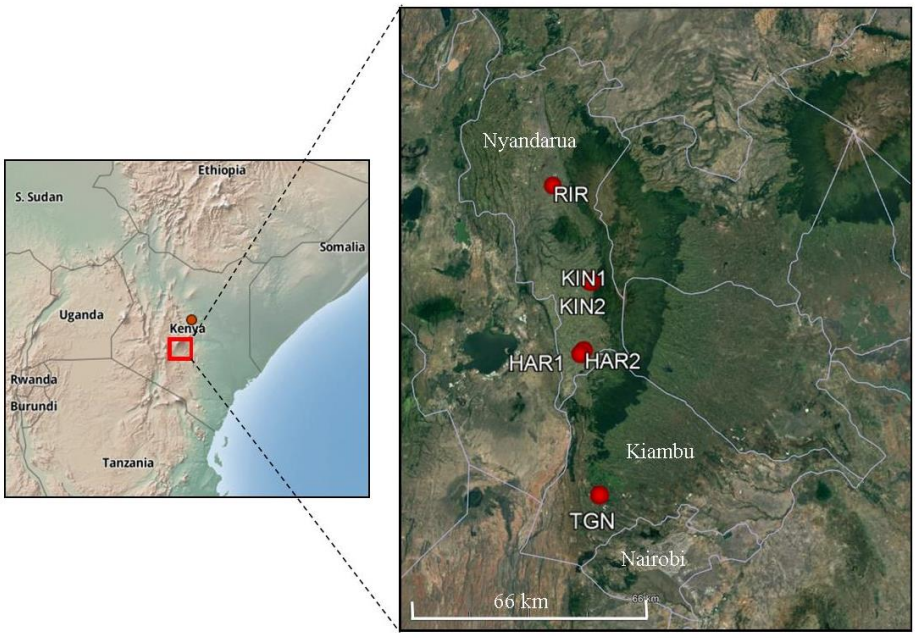

Figure 3.1 Map of Kenya indicating the locations (indicated by red dots) where the six *G. rostochiensis* populations were collected.

From each field, several soil samples were collected at different points and pooled into composite samples. These samples were thoroughly mixed and sub-samples of approx. 250 cm^3 were used for cyst extraction using a Fenwick can (Fenwick, 1940). Cysts were handpicked from the debris, packed and shipped to Julius Kühn Intitut, Braunschweig-Germany for identification and further analysis. The populations were reproduced twice on the susceptible potato cultivar 'Désirée' in the glasshouse before they were used in the study.

3.3.2 Species Identification by DNA Barcoding

The species identity of the six populations was confirmed by the amplification and sequencing of the mitochondrial cytochrome c oxidase subunit 1 (CO1) and the 28S rDNA large subunit (LSU) gene region (Holterman et al., 2006; Anonymous, 2016b). Genomic DNA was extracted from five single cysts from each of the six populations using QIAamp® DNA Micro kit (Qiagen GmbH-Germany) following the manufacturer's instructions.

DNA amplification was done with the primers listed in Table 3.1. The CO1 amplification was performed in a final volume of 25µl containing 9.9 µl MilliQ water, 12.5 µl HotStarTaq® Master Mix (Qiagen GmbH-Germany), 0.8 µl of each primer pair and 1 µl of DNA template. A positive

and a negative control were included in the run. The PCR program was set at 95 ºC for 5 min for initial denaturation, 40 cycles of denaturation at 95 ºC for 60 s, annealing at 41 ºC for 90 s, and extension at 72 ºC for 120 s and the final extension was done at 72 ºC for 10 min. The LSU amplification was also performed in a final volume of 25 µl containing 10.3 µl MilliQ water, 12.5 µl HotStarTaq® Master Mix (Qiagen GmbH-Germany), 0.6 µl of each primer pair and 1 µl DNA template. The PCR program was set at 95 ºC for 15 min for initial denaturation, 5 cycles of denaturation at 94 ºC for 30 s, annealing at 45 ºC for 30 s, and extension at 72 ºC for 30 s followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 52 ºC for 30 s, and extension at 72 ºC for 30 s and the final extension was done at 72 ºC for 5 min.

Table 3.1 Primers used for potato cyst nematode identification by CO1 and LSU DNA-barcoding[a]

Region	Name	Forward primer sequences (5'-3')[b]	Amplicon size	Reference
COI	JB3 (Forward)	TTTTTTGGGCATCCTGAGGTTTAT		
	JB5 (Reverse)	AGCACCTAAACTTAAAACATAATGAAAATG	400 bp	Hu et al., 2008
LSU	28-81(forward)	TTAAGCATATCATTTAGC GGAGGAA		
	28-1006 (reverse)	GTTCGATTAGTCTTTCGCCCCT	1000 bp	Holterman *et al.* 2008

[a]All primers were ordered online from Eurofins Genomics, Germany
[b]CO1 = cytochrome c oxidase subunit 1, LSU = 28S rDNA large subunit

PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GMBH & Co. KG, Germany) according to the manufacturer's instructions. The PCR products were then commercially sequenced by Macrogen Europe B.V. (Amsterdam, the Netherlands). Sequences obtained were assembled, edited and trimmed using Sequencher 5.4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). A BLAST search was used to check for the closely related sequences from the gene bank and Q-Bank EPPO (Bonants., 2013).

3.3.3 Microsatellite Genotyping

Genomic DNAs were extracted from 35 second-stage juveniles (J2s) coming from 35 distinct cysts per population. An individual juvenile per open cyst was picked under a dissecting microscope and transferred into a PCR tube containing 25 µl of lysis buffer constituted by 0.2 M NaCl, 0.2 Tris-HCl (pH 8.0), 1% (w/v) ß-mercaptoethanol, and 800 µg/ml proteinase-k (Holterman *et al*., 2006) and equal volume of MilliQ water. The tubes were incubated in a Thermocycler at 65 ºC for

2 h, followed by 95 ºC for 5 min. The lysate was centrifuged and the supernatant used in PCR reactions.

Microsatellite sequences were generated using the 12 primer pairs published by Boucher et al. (2013). Each primer pair had a fluorescent labelled forward primer for visualization of PCR products and a non-labelled reverse primer. The 12 markers were grouped into three multiplex combinations with four markers each. Multiplex 1 was made up of the following markers; Gr50, Gp109, Gp126 and Gp135, multiplex 2 had Gr85, Gr96, Gp116 and Gp118 while multiplex 3 was made up of Gr67, Gr75, Gr90 and Gr91.

The DNA template was diluted 1:4 and PCR performed in a 384 PCR well plate in 5 µl final volume reactions composed of 2.5 µl of 2X Type-it® Microsatellite PCR kit (Qiagen GmbH), 0.5 µl of 4 µM primer mix and 2 µl of a 4 times-diluted DNA template. The PCR programme was set at 95 ºC for 5 min for initial denaturation, 30 cycles of denaturation at 95 ºC for 30 s, annealing at 57 ºC for 90 s, and extension at 72 ºC for 30 s and the final extension was done at 60 ºC for 30 min. A negative control was included in the PCR amplification to check for potential contamination.

The amplified PCR products were diluted 1:40 in distilled water and 3 µl of the diluted DNA was sequenced as described by Boucher et al. (2013). The 3 µl diluted DNA was mixed with 0.05 µl of GeneScan^{TM} 500 LIZ® Size Standard (Applied Biosystems®) and 5 µl of formamide (Applied Biosystems®). Analysis of PCR products was conducted on an ABI Prism®3130xl sequencer (Applied Biosystems®) at INRA molecular lab in France. Allele sizes were identified using the GeneMapper® v4.1 Software (Applied Biosystems®) and the chromatograms were visually inspected to validate the results.

3.3.4 Data Analysis

Genetic linkage disequilibrium among the 66 pairs of markers was assessed by Markov approximation of the Fisher's exact test as implemented in GENEPOP 4.0.7 (Raymond & Rousset, 1995), and Bonferroni adjustment ($\alpha = 0.00076$) applied to correct the effect of multiple tests (Rice, 1989). To estimate the null allele frequency, each locus was tested using likelihood-based method (Chybicki & Burczyk, 2009) implemented in the INEst program.

The population gene diversity (H_{nb}) was estimated using GENETIX 4.05.2 (Belkhir et al., 2004) while the allelic richness (*Ar*), for a reduced sample size (*n* = 21), was estimated using POPULATIONS 1.2.32 (Langella, 1999). GENETIX was used to compute the deviation from random mating (F_{IS}) based on Weir & Cockerham (1984). The statistical significances of F_{IS} were estimated using the allelic permutation method (10,000 permutations).

To explore the genetic differentiation among populations, pairwise estimators of fixation indices (F_{ST}) were calculated according to Weir & Cockerham (1984) using GENEPOP 4.5.1 (Raymond & Rousset, 1995), and their statistical significances estimated by 5,000 random permutations of individuals among populations. A Bonferroni correction was applied to take into account multiple testing ($\alpha = 0.0033$ for 15 comparisons).

The genetic structure of the *G. rostochiensis* populations was explored using the six Kenyan populations and the 15 populations genotyped by Boucher et al. (2013), using the same set of 12 microsatellite markers. In the first step, a factorial correspondence analysis was performed, using GENETIX, on the 21 populations, and in the second step, the Bayesian model-based clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) was run on the 19 closest populations, excluding both Bolivian populations. This analysis was performed on a reduced dataset free of any missing data, including 374 individuals. Cluster (*K*) value was set from 1 to 19, and the calculation was repeated 20 times for each *K* value. The initial burn-in period was set to 1×10^6 iterations followed by 3×10^6 Markov chain Monte Carlo (MCMC) replications after burning. The calculations were done as suggested by Wang (2017): alpha was set to 0.053 and the uncorrelated allele frequency model was used. Structure Harvester (Earl & vonHoldt, 2012) was used to visualize the best K value based on ΔK (Evanno., 2005).

3.4 Results

3.4.1 Population Identification using DNA Barcoding

Blast of all the CO1 and LSU sequences generated in this study against gene bank sequences confirmed that the Kenyan populations used in this study were all *G. rostochiensis.* All six populations matched 99.8 to 100% with deposited DNA sequences.

3.4.2 Genetic Characteristics of the Populations

Among the six *G. rostochiensis* populations from Kenya, 33 to 35 individuals were successfully genotyped per population. After sequential Bonferroni's adjustment, only one locus pair, between Gp109 and Gp126, showed significant linkage disequilibrium out of the 66 marker pairs. When Kenyan populations were tested for null alleles by INEst, the percentage of potential null alleles ranged between 0% (Gp109, Gp118, Gr50 and Gr90) and 34% (Gp116) and averaged 5.7% among all 12-microsatellite markers.

The genetic diversity was quite similar among the Kenyan *G. rostochiensis* populations, except for the 'RIR' population, which was less diverse. The H_{nb} ranged from 0.113 ('RIR') to 0.205, and the *Ar* ranged from 1.760 ('RIR') to 2.211 alleles per population (Table 3.2). The deviation from random mating (F_{IS}) varied among the populations, ranging from -0.153 to 0.253 (Table 3.2). Two populations, 'HAR1' and 'HAR2', exhibited significant heterozygote deficit thus deviating from Hardy-Weinberg equilibrium (HWE). The two had high F_{IS} values of 0.137 and 0.253, respectively. The rest of the populations were at the Hardy-Weinberg equilibrium with F_{IS} not significantly different from zero (Table 3.2).

Table 3.2 Population gene diversity (H_{nb}), Allelic richness (*Ar*), and deviation from random mating (F_{IS}) in the six Kenyan populations of *G. rostochiensis*[a].

Population	No. sample (*n*)	H_{nb}	*Ar* (*n* = 21)	F_{IS}
'HAR1'	35	0.205	2.109	0.137*
'HAR2'	34	0.187	2.211	0.253*
'KIN1'	34	0.178	2.073	-0.012
'KIN2'	33	0.199	2.098	0.070
'TGN'	34	0.182	1.921	-0.025
'RIR'	34	0.113	1.760	-0.153

[a] n = number of individuals genotyped per population, H_{nb} = unbiased genetic diversity, *Ar* = allelic richness for reduced sample size (*n* = 21), F_{IS} = deviation from random mating. F_{IS} that are significantly higher than zero are indicated with asterisk.

3.4.3 Pairwise Genetic Differentiation

Significant pairwise genetic differentiations were detected between 'RIR' and the other populations (Table 3.3). The pairwise F_{ST} values for this population was $0.07 < F_{ST} < 0.11$. Differentiation was also detected between 'HAR1' and 'HAR2' ($F_{ST} = 0.06$) despite the two

populations being very close geographically. There was no significant genetic differentiation among all the other populations ($F_{ST} < 0.05$).

Table 3.3 Matrix of pairwise F_{ST} distances between Kenyan *G. rostochiensis* populations.

	'HAR1'	'HAR2'	'KIN1'	'KIN2'	'TGN'	'RIR'
'HAR1'	.					
'HAR2'	0.060*	.				
'KIN1'	0.048	0.013	.			
'KIN2'	0.047	-0.005	-0.007	.		
'TGN'	-0.002	0.042	0.014	0.023	.	
'RIR'	0.077*	0.110*	0.106*	0.111*	0.072*	.

* indicate a significant $F_{ST,}$ $P = 0.0033$ for 15 comparisons

3.4.4 Population Genetic Structure

In the factorial correspondence analysis the six Kenyan populations clustered together with the populations analysed by Boucher et al. (2013) except for two populations from Bolivia that formed separate clusters (Fig. 3.3a).

Excluding both Bolivian populations (B2 and B4), the number of genetic clusters corresponding to the highest Evanno's Δ*K*' probability was clearly $K = 2$ clusters in the Bayesian clustering analysis (Fig. 3.2). The Kenyan populations formed one cluster while populations from Boucher et al. (2013) formed a separate genetic cluster (Fig. 3.3b). With the increase in *K* value from 2 to 4, the Kenyan populations did not show further population differentiation while the populations from Boucher et al. (2013) formed three subgroups (Fig. 3.3c), corresponding to the three clusters described previously (Boucher et al., 2013)

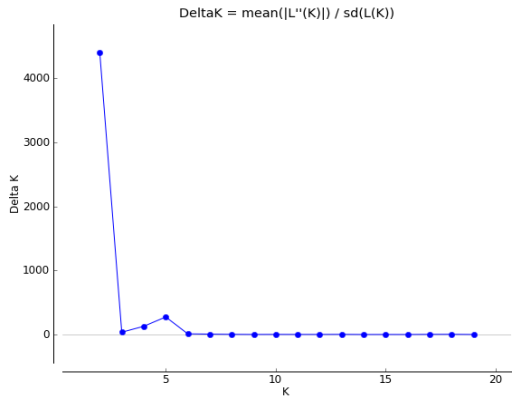

Figure 3.2 Plot of Δ*K* used to determine the most likely number of genetic structure (*K*) of Kenyan and Boucher et al. (2013) *G. rostochiensis* populations.

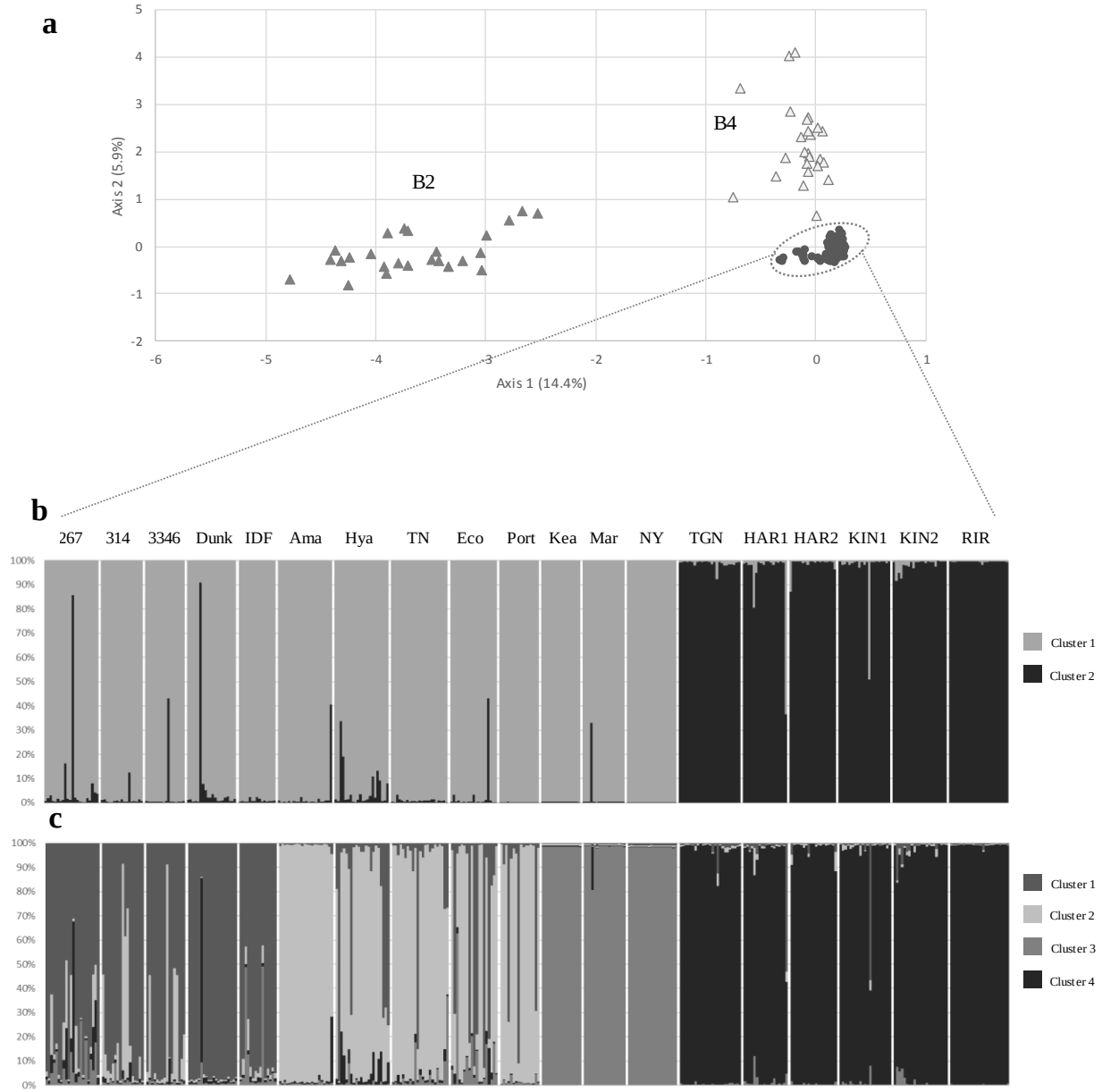

Figure 3.3 Genetic structure of *G. rostochiensis* populations from Kenya ('HAR1', 'HAR2', 'KIN1', 'KIN2', 'TGN' and 'RIR'); Europe-France ('Dunk' & 'IDF'), Scotland ('Eco') and Portugal ('Port'); South America- Bolivia ('B2' & 'B4'), Peru ('267' & '314') and Chile ('3346') and North America-Canada ('Ama', 'Hya', 'TN', 'Kea' & 'Mar') and United States ('NY') based on the microsatellite genotyping. a) Result from the factorial correspondence analysis: Bolivian populations ('B2' and 'B4') are genetically distinct from all the other populations; b) Results from the Bayesian clustering analysis (STRUCTURE) for $K = 2$; c) Results for $K = 4$. Each vertical line represents an individual for which the genetic assignment is partitioned into two ($K = 2$) or four ($K = 4$) clusters. Vertical white lines separate each of the 19 populations.

3.5 Discussion

Using CO1 and LSU DNA barcoding, all the six PCN populations used in this study were identified as *Globodera rostochiensis*. The findings confirm the identity of PCN *G. rostochiensis* in Kenya as reported by Mwangi et al. (2015).

Only two populations ('HAR1' and 'HAR2') out of six populations from Kenya showed a heterozygote deficit, the rest were at Hardy-Weinberg equilibrium (HWE). Presence of null alleles could contribute to heterozygote deficit in a population genetic analysis (Montarry., 2015). However, in this study only a single marker out of twelve markers used had potential null alleles. Moreover, heterozygote deficit is common with cyst nematodes: it was highlighted for *Globodera pallida* (Picard et al., 2014), *Heterodera schachtii* (Plantard & Porte, 2004; Kim et al., 2018), *H. glycines* (Wang et al., 2015) and *H. carotae* (Gautier et al., 2019), among others. Boucher et al. (2013) found significant heterozygote deficit in only three out of 15 *G. rostochiensis* populations. In the present study, four Kenyan populations were at HWE. This is not typical of cyst nematodes, which have limited dispersal ability leading to high inbreeding.

A bottleneck arising from recent introduction(s) of these populations can explain the low genetic diversity and allelic richness reported. Population disturbances such as introduction into new environment creates a genetic bottleneck that eliminates low frequency alleles from the population leading to the loss of variability (Wright, 1931; Nei., 1975; Templeton, 1980). Such a loss is to the disadvantage of the population that needs to adapt to the new habitat. Thus, It takes time for the population to increase and recover the lost alleles through mutation and random mating (Prakash., 1969). Recovery from such a bottleneck depends on the rate of population growth that follows (Nei et al., 1975). With *G. rostochiensis* being reported in Kenya only a few years ago (Mwangi et al. (2015), it is possible that the populations have not yet recovered from the bottleneck of the founder effect (Nei et al., 1975).

In the matrix of pairwise F_{ST} distance comparison, Kenyan *G. rostochiensis* showed low genetic differentiation as manifested by the low distance values in four out of the six populations. Such differentiation is often attributed to significant gene flow, which in this case may arise from human-mediated passive dispersal or natural soil movement between farms (Plantard & Porte 2004; Alenda et al., 2014). Human assisted dispersal of PCN highly facilitates the spread of

nematodes at the regional scale (Banks et al., 2012). For instance, common farm practices among the smallholder farmers in Kenya include recycling and sharing of seed potatoes among farmers (Gildemacher et al., 2011), sharing of farm implements, and transportation of potato tubers without cleaning the adhering soil, among others.

It has been speculated that the Kenyan populations are of European descent and that they were introduced into the country within the last three decades because a study done in Kenya in the early 1980s failed to find PCN species (Njoroge, 1982). Surprisingly, once the Bolivian populations were removed from the analysis, the Kenyan populations did not cluster closely with the European populations included in the analyses (Boucher et al., 2013). The STRUCTURE analysis generated two genetic structures. Kenyan populations formed one solid structure while the rest of the foreign populations formed another structure. There was no further population subdivision in Kenyan populations when increasing the *K* value. However, the Boucher et al. (2013) populations segregated further into three substructures at $K = 4$. The question of the source population and the route of entry of the Kenyan populations remain thus open.

Considering the unique cropping system in Kenya, with up to three crop cycles per year with little or no rotation (Muthoni et al., 2013) and no diapause (see Chapter 2), nematodes are able to complete three or more generations per year as opposed to one generation in temperate countries (Moens et al., 2018). Consequently, the nematode population densities likely increase rapidly (Greco, 1993) leading to early detection. The introduction of *G. rostochiensis* in Kenya may have happened relatively recently.

Although the Kenyan *G. rostochiensis* belong to the Ro1/4 pathotype group (see Chapter 2) which is the predominant pathotype in Europe, the current study could not confirm the relationship between the Kenyan populations and the European populations, probably because there were no representative samples of all European populations for comparison. Nevertheless, the association between the Kenyan populations and the European and American populations is closer compared to the Bolivian populations. Two hypotheses have been proposed to explain this: first, Kenyan populations may have originated from a European population that has not yet been genotyped. Indeed, Kenya has a long history of importing seed potatoes from Europe (Njoroge, 1982; Anonymous, 2016a). Populations from European countries that supply seed potatoes to Kenya

were not included in the Boucher et al. (2013) work and therefore could not be analysed in this study. The second hypothesis is that the introduction of *G. rostochiensis* was from another source not yet established. This may be the case as Kenyan populations had a higher degree of homogeneity among them compared to the other populations. To test the two hypotheses a more detailed study is required to compare more collections of *G. rostochiensis* from countries in Africa and Europe from which seed potatoes are imported.

The knowledge about the genetic diversity of PCN populations has implications for the deployment of resistant cultivars in pest management (Picard et al., 2004). Improper utilization of resistant cultivars may induce selection leading to emergence of more virulent populations (Picard et al., 2004; Fournet et al., 2013) or an increase of *G. pallida* that was reported in the Nyandarua region (Mburu et al., 2018). This happened in the UK where introduction of potato cultivars with full resistance to *G. rostochiensis* led to the replacement of this species by *G. pallida* (Minnis et al., 2002; Hockland et al., 2012). However, given the low genetic diversity reported in this study, the deployment of resistant cultivars should significantly reduce the reproduction.

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Chapter 4: Reproduction and Life History Traits of a Resistance Breaking *Globodera pallida* Population

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4.1 Abstract

The main and most efficient measure to control potato cyst nematode (PCN) is the use of resistant cultivars. German and Dutch National Plant Protection Organizations (NPPOs) recently reported the emergence of *Globodera pallida* populations virulent on potato cultivars carrying resistance against pathotype 2/3. The development and virulence of the virulent population 'Oberlangen' from Germany in comparison to the reference population *G. pallida* Pa3 'Chavornay' were investigated on resistant and susceptible cultivars in glasshouse experiments. Various life history traits associated with change in virulence were also assessed. Hatching of second-stage juveniles (J2s) was similar for both populations but incubation of cysts in potato root diffusate resulted in higher hatching rates compared to 3 mM Zinc Chloride and tap water. Both populations showed high penetration rates in the roots of the resistant and susceptible cultivars. However, only the population 'Oberlangen' was able to complete its life cycle in the roots of the resistant potato cultivar. In 'Seresta', the resistance response restricted the formation of females by avirulent individuals in favour of males. 'Oberlangen' was virulent on all cultivars tested. No difference in cyst size, number of eggs per cyst, length of juveniles and males was found for 'Oberlangen' and 'Chavornay' on the susceptible cv. 'Désirée'. However, cysts of virulent populations from the same region 'Oberlangen' was obtained from had a significantly larger diameter compared to avirulent populations. The population 'Oberlangen' showed a higher reproduction and fitness than the reference population 'Chavornay' on susceptible cultivars and could serve as a future reference population in testing of new potato cultivars for resistance against this new virulence type in Europe.

Keywords
Biology, *Globodera pallida, G. rostochiensis,* hatching, life-history traits, pathotype, resistance, virulence selection

4.2 Introduction

The potato cyst nematode (PCN) *Globodera pallida* (Stone, 1972) is a potato pest of economic importance worldwide. Plant resistance is the most efficient way to reduce crop damage and reproduction of this nematode. In contrast to *Globodera rostochiensis* (Wollenweber, 1923) which is effectively controlled using resistant potato cultivars, there is a lack of cultivars with stable resistance to *G. pallida* pathotypes. Nevertheless, using plant resistance in an integrated pest

management system remains the most viable way to keep nematode densities below the damage threshold. European populations of *G. pallida* are highly heterogeneous (Turner et al., 1983; Turner & Fleming, 2002; Dalton et al., 2013; Rigney et al., 2017) mainly consisting of pathotype 2 and 3 (Pa2/3). As a consequence, no single resistance gene is able to confer full resistance against all European populations of *G. pallida* Pa2/3 (Dalton et al., 2013). The potato cultivars available to the growers have been introgressed with resistance derived from species such as *Solanum vernei* (*Gpa1* & *Gpa5*), *S. tuberosum* spp. *andigena* (*H1* & *Gpa2*), and *S. spegazzinii* (*Gpa*), among others (Bakker et al., 2006; Dalamu et al., 2012). These genes confer pathotype specific resistance and they differ in their mode of action.

Infestation of potato plants by *G. pallida* occurs soon after the hatching of second-stage juveniles (J2s), a process that is initiated by chemical stimuli present in the potato root exudate. The exudate is continuously produced by actively growing potato roots and diffuses in the rhizosphere where it comes into contact with nematode cysts. Hatched J2s then locate young host roots and penetrate near the root tip. They move intracellularly towards the pericycle and use a stylet to pierce the cell walls and inject salivary secretions containing growth regulators into the host cells. These regulators function by modifying the host cells into a feeding structure known as syncytia that ensures continuous nourishment of the developing nematode (Moens et al., 2018).

The presence of the invasive juveniles in the roots of resistant hosts trigger a cascade of immune responses that thwart further development of the nematodes. Potato cultivars having different resistance gene(s) respond differently to PCN infestation. The J2s fail to initiate syncytia and therefore exit or die within the root. In other cases, development of the nematode in the host root may be arrested or there may be a shift in sex ratio in favour of males (Trudgill, 1967; Rice et al., 1985; Schouten, 1993; Bakker et al., 2006; Williamson & Kumar, 2006; Smant et al., 2018). For instance, the *H1* gene which confers resistance to pathotype Ro1 and Ro4 of *G. rostochiensis* triggers a hypersensitive reaction characterized by necrosis and death of cortical cells surrounding the invading nematode (Rice et al., 1985; Bakker et al., 2006; Smant et al., 2018). This is followed by the formation of a syncytial complex which restricts the development of the syncytium resulting in poorly formed feeding cells. The invading J2s are deprived of adequate food leading to the formation of more males instead of females since sex in PCN is epigenetically determined (Trudgill, 1967; Schouten, 1993; Bakker et al., 2006). In plants having GPa2 resistance genes,

syncytia may be formed, but proliferation is arrested within a few days. The cells surrounding the syncytium become necrotic leading to the degeneration of the feeding structure. In this case, females are formed but their development is arrested and they fail to develop eggs (Bakker et al., 2006).

When cultivars carrying identical resistance genes are grown repeatedly for several generations, they impose strong selection pressure which increases the frequency of virulent individuals within a nematode population (Turner, 1990; Turner & Fleming, 2002). There are reports for Germany and The Netherlands about *G. pallida* populations with the new virulence type due to overuse of potato cultivars carrying quantitative resistance genes (Niere et al., 2014). Resistance breaking has been reported in other important plant parasitic nematodes such as *Meloidogyne incognita* overcoming the *Mi* gene in tomatoes (Kaloshian et al., 1996).

Change of virulence of *G. pallida* populations has been studied in controlled experiments (Turner et al., 1983; Turner, 1990; Beniers et al., 1995; Schouten & Beniers, 1997; Turner & Fleming, 2002; Beniers et al., 2019). In this case, isolates with increased virulence were artificially selected from avirulent populations reared on hosts carrying resistance genes (Schouten & Beniers, 1997; Castagnone-Sereno et al., 2007; Fournet et al., 2013). Schouten & Beniers (1997) multiplied *G. pallida* Pa3 on resistant cultivar 'Karakter' and they noted a significant increase in virulence after three generations on the same cultivar. Turner et al. (1983) detected a change in virulence of *G. pallida* after five generations of reproduction on PCN resistant *S. vernei* hybrids while in a study carried out by Fournet et al. (2013) it took the nematode eight years to completely overcome host resistance.

Change of virulence of a population as a result of selection on a resistant cultivar is often associated with a fitness cost (Thrall, 2003). This is a penalty that comes in form of reduction in pathogenic aggressiveness in a susceptible host or a compromise on another trait (Vera Cruz et al., 2000). This has been confirmed with bacteria (Ferenci, 2016; Peyraud et al., 2016), some fungi (Montarry et al., 2010), viruses (Jenner et al., 2002) and some plant parasitic nematodes. For instance, Castagnone-Sereno et al. (2007) found that *Meloidogyne incognita* selected on tomato carrying the *Mi* resistance gene had a lower fitness on susceptible hosts. In contrast, Turner, (1990), reported no compromise in fitness of *G. pallida* despite several generations of selection on resistant cultivars. Bernier et al. (1995) reported that increase in virulence of *G. pallida* is associated with

increased fitness on a susceptible host. This was confirmed by Fournet et al. (2016), when they studied the life-history traits of a *G. pallida* lineage that had been selected on resistant potato cultivars. They found that the lineage formed bigger cysts with more eggs on the susceptible cultivar and hatched faster compared to the unselected lineages. No such study has been done with *G. pallida* populations selected in the field.

Recently, Niere et al. (2014) reported a new virulence type of *G. pallida* in populations obtained during field surveys in the Emsland region of Lower Saxony, Germany. This new virulence type, herein referred to as population 'Oberlangen', was able to reproduce on starch potato varieties carrying resistance genes against pathotypes Pa2/3. 'Oberlangen' is an example of selection by continued cultivation of resistant potato cultivars against *G. pallida* Pa2/3. However, there is little information regarding this population and other virulent field populations from the same region.

In this study, the 'Oberlangen' population was selected for further testing and characterization using *G. pallida* 'Chavornay' (Anonymous, 2006) as a reference population. This study aimed at comparing the development, virulence and fitness of *Globodera pallida* 'Oberlangen' to the *G. pallida* 'Chavornay' and to assess various life-history traits associated with change in virulence.

4.3 Materials and Methods

4.3.1 Nematode Populations

Globodera pallida 'Oberlangen' and *G. pallida* 'Chavornay' were used in the study. The origin of 'Oberlangen' are potato fields in Emsland region of Lower Saxony, Germany (Niere et al., 2014) while 'Chavornay', which is the official reference population used in testing potato cultivars for resistance against *G. pallida* Pa3 (Anonymous, 2006), was obtained from the JKI-Braunschweig-Germany. The two populations were maintained on the susceptible cv. 'Désirée' in JKI-Braunschweig. Prior to the study, the populations were reproduced and subsequently stored at 4 °C for a minimum period of six months to break diapause.

Plant Material

Potato cultivars with different levels of resistance to *G. pallida* Pa2/3 (Anonymous, 2017) were used in this study. The cultivars 'Laura', 'Albatros' and 'Belana' lack resistance to *G. pallida*, but they are highly resistant to *G. rostochiensis* Ro1/4. Potato cultivar 'Ribera' has a partial resistance to *G. pallida* (score = 6), but the source of resistance could not be established. The cultivars, 'Amado', 'Amanda' and 'Seresta' are rated high in their resistance to *G. pallida*. The three cultivars carry several resistance genes largely from *Solanum vernei* and *S. tuberosum* ssp. *andigena* (Hutten & Berloo, 2001; van Berloo *et al*., 2007). The source of resistance in 'Euroviva' and 'Eurotonda' is assumed to come from *S. vernei*, but this information is not publicly available. Potato cultivar 'Désirée' lacks resistance to PCN and therefore it was used as the reference cultivar. The potato tubers were pre-germinated in the dark at room temperature before transferring the sprouting tubers into a well-lit room for shoot hardening.

4.3.2 Hatching Assay

Cysts of both *G. pallida* populations 'Oberlangen' and 'Chavornay' were incubated in potato root diffusate (PRD), Zinc Chloride ($ZnCl_2$) or tap water (H_2O). The hatched J2s were counted weekly for a period of eight weeks. PRD was obtained from the susceptible potato cv. 'Désirée' grown in the glasshouse for a period of three weeks. The plants were uprooted and roots cleaned in running water to remove adhering soil and placed in a beaker with the roots suspended in 250 ml tap water. The setup was left in the dark overnight (Rawsthorne & Brodie, 1986). Thereafter, the root diffusate was filtered using filter paper (Macherey-Nagel GmBh & Co. KG). Collected PRD was diluted by adding equal volume of water and stored at 4 ºC for a short period prior to use, fresh PRD was continuously produced for the entire period of the experiment. A standard concentration of 3 mM of $ZnCl_2$ (Greet, 1974) was used alongside PRD and tap water.

Hatching assays were conducted using batches of 20 cysts from the same reproduction cycle using four replications per treatment. Hatching was done in tubes measuring 15 mm in diameter and 60 mm height with a 100 µm sieve fixed at the bottom to hold the cyst, but allow movement of hatched juveniles into the hatching media. Hatching tubes containing cysts were placed into 15 ml Falcon tubes filled with hatching medium. Cysts were soaked in tap water for a week before they were transferred into the hatching media and incubated in the dark at room temperature. The hatched juveniles collected at the bottom of the tubes were counted weekly and the hatching media

renewed. Hatching was monitored over a period of eight weeks after which the cysts were crushed and the number of unhatched eggs determined.

4.3.3 Development of 'Oberlangen' and 'Chavornay' in Potato Roots

The development of *Globodera pallida* 'Oberlangen' in the roots of susceptible cv. 'Désirée' and resistant cv. 'Seresta' was studied and compared with the reference population *G. pallida* 'Chavornay'. Eye-plugs were scooped from pre-sprouted tubers using a melon baller and planted in 192 ml pots containing loess soil enriched with slow release fertiliser ((Osmocote Exact Standard®) 15% N, 9% P_2O_5,12% K_2O and 2% MgO) at a rate of 1.5 g (kg soil)$^{-1}$. (Müller & Rumpenhorst, 2000; Mwangi, et al., 2019). One hundred and twenty pots containing either cv. 'Désirée' or cv. 'Seresta' were inoculated with hatched J2s of 'Oberlangen' or 'Chavornay', respectively. To obtain inoculum, nematode cysts were put on a 250 µm plastic sieve placed on a funnel with a tube clamped at the bottom. The cysts were soaked in water for one week after which the water was replaced with PRD produced as described above. The setup was left in the dark at room temperature to induce hatching. After seven days, hatched juveniles were enumerated and used for inoculation. Inoculation was done 14 days after planting. Sixty pots with cv. 'Désirée' and 60 'Seresta' plants were inoculated with J2s of either 'Oberlangen' or 'Chavornay' population. For inoculation, two-30 mm deep holes were made in the moist soil using a plastic rod and approximately 600 J2s were dispensed equally into the two holes using a pipette and the holes carefully covered with soil. Pots were completely randomized in a metal box and placed on the glasshouse bench. Plants were watered as required throughout the experiment. Glasshouse temperatures were set at 18 ± 2°C. Soil and air temperature were recorded hourly using a Testo® 175T3 (Testo Ltd, UK) temperature logger.

Seven days post inoculation (DPI), and weekly thereafter, four pots from each treatment were randomly picked and used to assess the development of nematodes in the roots. Plants were removed from the pot and the soil clinging to the roots carefully collected for the extraction of J2s and males. Roots were then rinsed in running water and the entire root system stained with acid fuchsin (Byrd et al., 1983). Stained nematodes were visually examined under the Nikon® SMZ1270 stereo microscope and nematodes at different stages of development recorded. Male nematodes were extracted from the soil using the centrifugation flotation method (Anonymous 2013).

4.3.4 Assessing the virulence of 'Oberlangen' and 'Chavornay'

The reproduction of *G. pallida* 'Oberlangen' and 'Chavornay' was assessed on the ten potato cultivars listed above. For each of the cultivars, small sized tubers were planted in 1,000 ml pots using loess soil as a substrate. Upon emergence, (approx. 2 wks. after planting) five pots each were inoculated with 5 eggs and J2s ml^{-1} soil (Anonymous, 2006) of either 'Oberlangen' or 'Chavornay'. Inoculum was prepared by soaking cysts in water and crushing them to free eggs and J2s (Seinhorst & Den-Ouden, 1966). The eggs and J2s in the suspension were counted and adjusted to achieve an estimated number of 500 eggs and J2s ml^{-1}. The eggs and J2s suspension was dispersed into four 30 mm deep holes made into the substrate to achieve the initial density (P_i) of 5 eggs and J2s ml^{-1} soil. The holes were then carefully covered with soil and the pots randomized on the glasshouse bench. The air and soil temperatures were recorded throughout the experiment as described above.

Twelve weeks after inoculation, the experiment was terminated and cysts extracted from each pot by washing the soil through a 250 µm bucket sieve (Mwangi et al., 2019). Cysts and plant debris retained in the sieve were collected on filter paper and the content dried at 35 °C for 3 days. Cysts were then separated from the plant debris using acetone (van Bezooijen, 2006) and counted under a stereo microscope. To determine the final nematode population (P_f) and the reproduction factor (R_f), all the extracted cysts per pot were crushed and the average number of eggs per cyst estimated. All the experiments were repeated once.

4.2.5 Estimating the Size of the Cysts and the Number of Eggs per Cyst

The size of the cysts of *G. pallida* 'Oberlangen' and 'Chavornay' extracted from the susceptible cv. 'Désirée' and resistant cv. 'Seresta' were measured using a Nikon® SMZ18 Stereo Zoom Microscope. Measurements were taken of forty randomly picked cysts per population and cultivar. In addition to measuring the diameter, the cyst content was also determined in batches of ten cysts replicated ten times, to estimate the mean number of eggs and J2s per cyst (Seinhorst & Den-Ouden, 1966; van Bezooijen, 2006).

The size of the cysts of six other virulent populations (NI-Gpa-VIR002, NI-Gpa-VIR003, NI-Gpa-VIR004, NI-Gpa-VIR011, NI-Gpa-VIR012 & NI-Gpa-VIR013 and three avirulent populations (NI-Gpa-AVI001, NI-Gpa-AVI002 & NI-Gpa-AVI003) was determined as described above.

These populations had been obtained at different time period during field surveys in the Emsland region of Lower Saxony-Germany and their virulence determined (S. Kruessel, LWK-Niedersachsen, pers. comm.). However, unlike 'Oberlangen', they were not reproduced in the glasshouse prior to the study.

4.2.6 Estimating the Size of J2s and Males

The males of *G. pallida* 'Oberlangen' and 'Chavornay' recovered from the susceptible cv. 'Désirée' and resistant cv. 'Seresta' were measured. Male nematodes were handpicked from a water suspension and mounted on a microscopic slide with a drop of water. A cover slip was carefully placed on the drop of water containing nematodes and the slide placed briefly on a hot plate at 60 °C to relax the nematode. Measurements were taken from 40 males per treatment using a Nikon® SMZ18 Stereo Zoom Microscope. To measure the J2s, cysts recovered from the susceptible cv. 'Désirée' and resistant cv. 'Seresta' used in the development study above were placed in PRD to induce hatching. J2s were then handpicked and temporary slides prepared as above. Measurements were taken of 40 J2s per treatment.

4.2.7 Data Analysis

The reproduction factor (R_f) of the nematodes was determined by dividing the final nematode population (P_f) by the initial population (P_i). The relative susceptibility (R_s) of the tested potato cultivars and their levels of resistance to 'Oberlangen' and 'Chavornay' were determined as described in the EPPO (Anonymous, 2006) using cv. 'Désirée' as a susceptible reference control. Data were tested for normality using Shapiro test while Levene's test was used to assess the homogeneity of variance. Analysis of variance was done for data on size of cysts, J2s and males as well as the mean number of eggs per cyst. Means that were significantly different ($P \leq 0.05$) were separated using Tukey's HSD test. There was no significant difference ($P > 0.05$) between two experiments testing the virulence of *G. pallida* 'Oberlangen' and 'Chavornay'. Therefore, data from the two experiments were pooled prior to analysis. T-test was used to compare the mean number of cysts per cultivar between 'Oberlangen' and 'Chavornay'. Kruskal-Wallis test was used to compare mean number of cysts per cultivar within each of the two populations as well as comparing the cyst diameter of the virulent and avirulent populations. Means that were significantly different at $P \leq 0.05$ were separated using Kruskal Post Hoc test. All statistical

analyses were done using R-software version 3.6.0 (R Foundation for statistics computing) and data were plotted using SigmaPlot® 13.0.

4.4.0 Results

4.4.1 Hatching Assays

The number of hatched juveniles of *Globodera pallida* 'Oberlangen' and *G. pallida* 'Chavornay' over 8 weeks was greater in PRD ($P < 0.05$) than in $ZnCl_2$ and the control (Fig. 4.1). Except for the control, the percentage of hatched juveniles in PRD was significantly greater for 'Chavornay' compared to 'Oberlangen' between week one and four ($P < 0.05$). After 8 weeks, the proportion of hatched juveniles was higher in 'Chavornay' with 67.78% compared to 'Oberlangen' with 62.80%. Hatching in H_2O was lower than in PRD and $ZnCl_2$ for both populations.

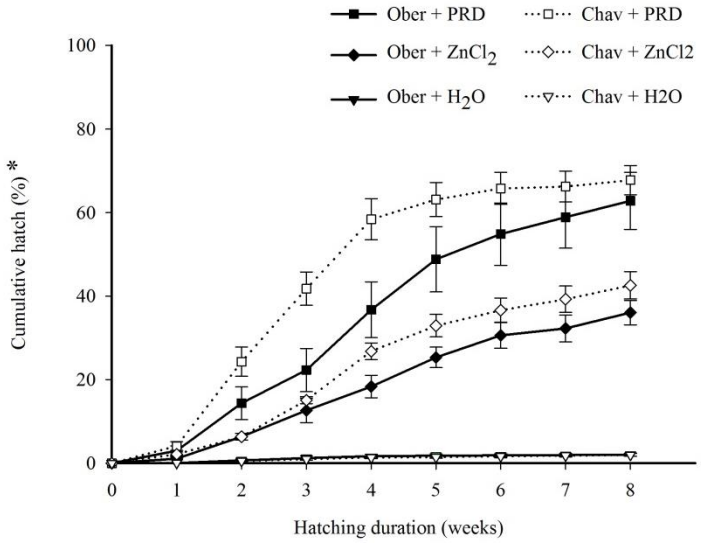

Figure 4.1 Cumulative percentage hatch of *Globodera pallida* 'Oberlangen' (Ober) and 'Chavornay' (Chav) in potato root diffusate (PRD), Zinc Chloride ($ZnCl_2$) and water (H_2O) over a period of 8 weeks. The error bars represent the standard error of the mean.

4.4.2 Development of 'Oberlangen' and 'Chavornay' in Potato Roots

In both experiments, invasive juveniles of the two nematode populations were found in similar numbers at 7 DPI in the roots of the susceptible and resistant cultivars (Fig. 4.2). However, in the first experiment, the average number of J2s that penetrated the host within seven days was higher with 141 ± 20 J2s per root system, compared to the second experiment with 81 ± 10 J2s per root system. The number of J2s in the roots decreased significantly during the first three samplings. At 14 and 21 DPI, the number of 'Oberlangen' and 'Chavornay' J2s in the resistant cv. were higher

($P < 0.05$) than in the roots of the susceptible cv. in experiment one (Fig. 4.2). In the second experiment, J2s of 'Oberlangen' were still found in roots of cv. 'Seresta' at 35 DPI while only few J2s were detectable in 'Désirée' roots. The number of 'Chavornay' J2s moulting into the progressive stages in cv. 'Seresta' was lower compared to 'Oberlangen' (unpubl.data).

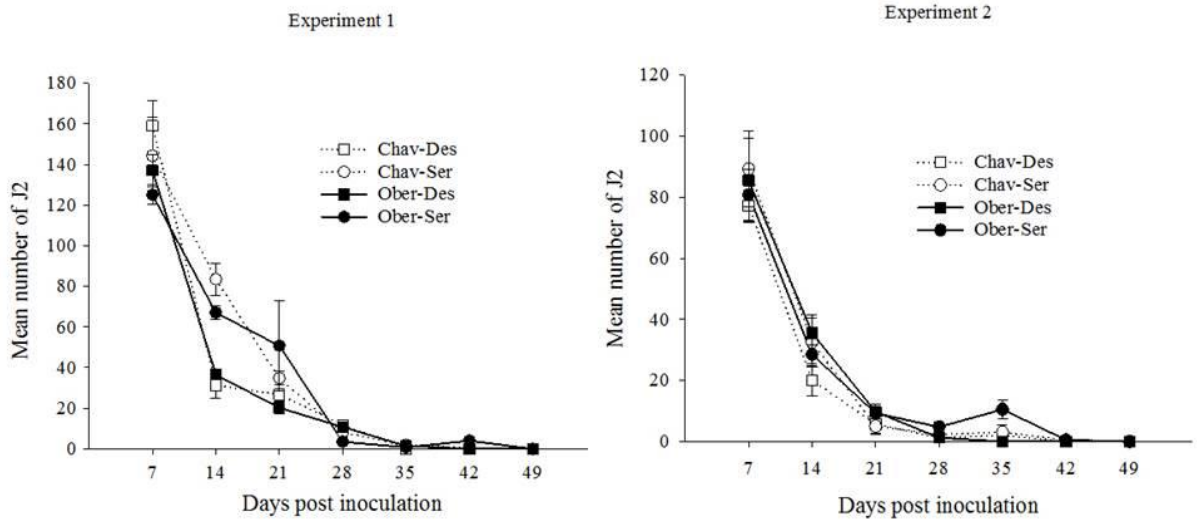

Figure 4.2 Number of second-stage juveniles (J2s) of *Globodera pallida* 'Oberlangen' (Ober) and 'Chavornay' (Chav) in the roots of the susceptible cv. 'Désirée' (Des) and resistant cv. 'Seresta' (Ser) potato cultivars 49 days after inoculation. Four plants ($n = 4$) of each nematode-cultivar combination were sampled and J2s enumerated at different days post inoculation in experiment one and two. The vertical bars represent the standard error of the mean.

Male nematodes were detected in the roots of 'Désirée' and 'Seresta' at 21 DPI in both experiments (Fig. 4.3). There was no significant difference in the number of males of the two populations recovered from the roots of the resistant cultivar 'Seresta'. However, for 'Désirée', 'Chavornay' produced more males than 'Oberlangen'. Subsequently, the number of males recovered from the soil increased significantly reaching the climax at 35 DPI. In the first experiment, the number of males recovered on the resistant cv. between 21 and 56 DPI was significantly higher ($P < 0.05$) compared to the susceptible cultivar. In the second experiment, 'Oberlangen' had a higher number of males on the resistant cultivar ($P < 0.01$) compared to 'Chavornay' (Fig. 4.3). On the susceptible variety, the number of males remained low in both experiments. The trend remained the same until 56 DPI when males were rarely detected in the soil.

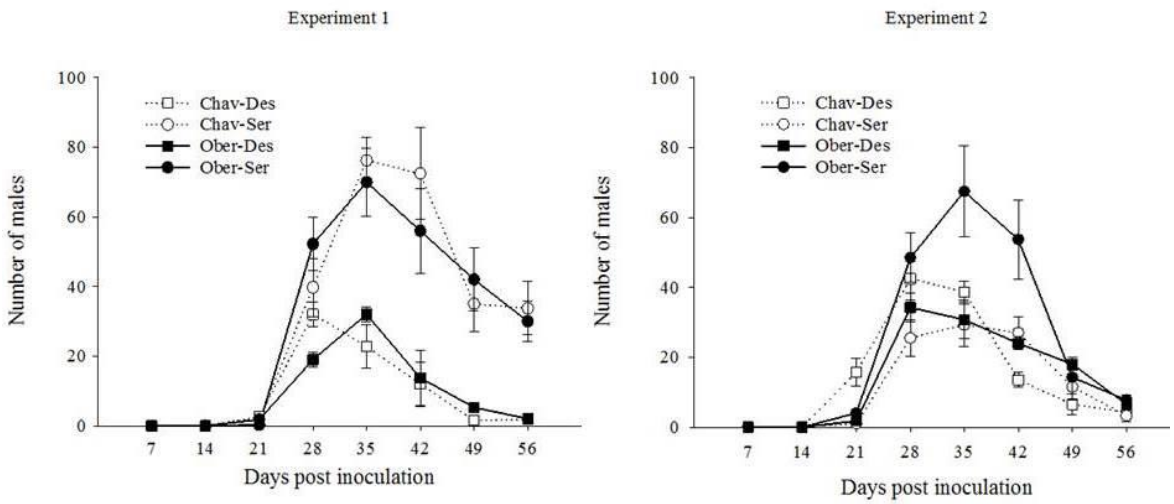

Figure 4.3 Numbers of male nematodes of *Globodera pallida* 'Oberlangen' (Ober) and 'Chavornay' (Chav) in the roots and soils of the susceptible cv. 'Désirée' (Des) and the resistant cv. 'Seresta' (Ser) potato cultivars 56 days after inoculation. Four plants (n = 4) in each treatment were sampled and the males enumerated at different days post inoculation in experiment one and two. The vertical bars represent the standard error of the mean.

The first young female nematode was recorded in plant roots at 21 DPI in both experiments (Fig. 4.4). The number of females of the two populations in the roots of 'Désirée' and 'Seresta' differed significantly throughout the experiment ($P < 0.05$). Between 21 and 42 DPI, there was an increase in the number of females on the susceptible cultivar. However, on the resistant cultivar, females were found in plants inoculated with 'Oberlangen', but rarely in the roots of cv. 'Seresta' for 'Chavornay'. There was a higher number of 'Oberlangen' females on cv. 'Désirée' compared to 'Chavornay' in the second experiment (Fig. 4.4).

However, the mean number of females of the two populations did not differ in both experiments. The first brown cyst was recorded at 42 DPI on the susceptible cultivar and at 49 DPI on the resistant cultivar, respectively (unpubl.data). During the first experiment, nearly all females had turned brown 56 DPI and in the second experiment one week later. There was no difference in the duration taken by the two populations to complete the life cycle although 'Oberlangen' females exhibited a prolonged white stage compared to 'Chavornay'.

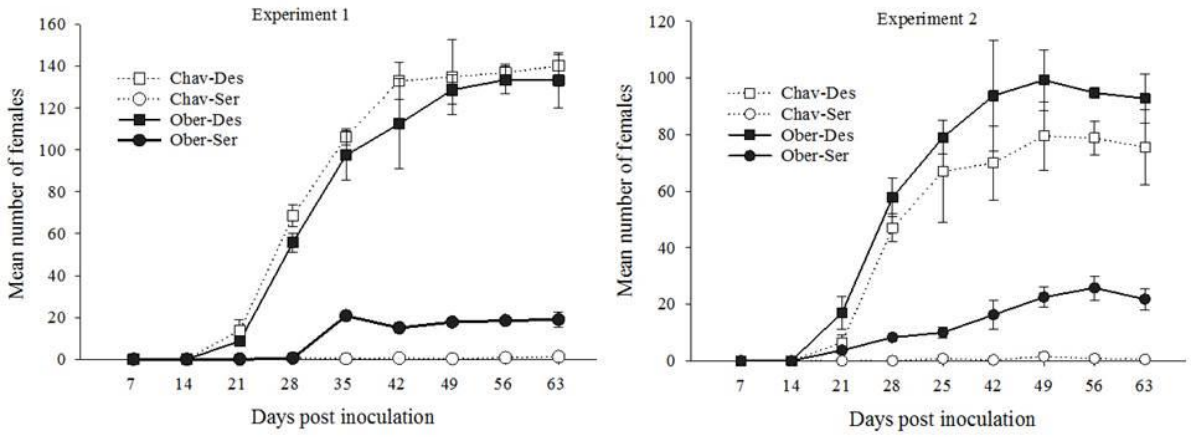

Figure 4.4 Numbers of females of *Globodera pallida* 'Oberlangen' (Ober) and 'Chavornay' (Chav) on the roots of the susceptible cv. 'Désirée' (Des) and the resistant cv. 'Seresta' (Ser) potato cultivars. Four plants (n = 4) in each treatment were sampled and females enumerated at different days post inoculation in experiment one and two. The vertical bars represent the standard error of the mean.

4.4.3 Assessing the virulence of 'Oberlangen' and 'Chavornay'

The reproduction of *Globodera pallida* 'Oberlangen' and 'Chavornay' was highest on the susceptible cv. 'Désirée' (Table. 4.1). The mean number of cysts on the susceptible cv. was not different between the two nematode populations ($P > 0.05$). This cultivar was therefore used as the standard susceptible control and the mean number of cysts recovered from it was used in the calculation of relative susceptibility of the tested potato varieties. The number of 'Oberlangen' cysts as well as the 'Chavornay' cysts extracted from the tested potato cultivars varied significantly (($P < 0.01$) Table 4.1).

Except for 'Désirée', 'Albatros', 'Laura' and 'Belana', 'Oberlangen' produced significantly more cysts than 'Chavornay'. 'Albatros' and 'Laura' potato cultivars were highly susceptible to the 'Oberlangen' population with R_s of 69.0% and 63.3%, respectively. In contrast, the rest of the cultivars had R_s values of 50% and below, but only 'Amanda' was below 10%. Potato cv. 'Albatros' and 'Laura' were highly susceptible to 'Chavornay' with R_s of 63.2% and 53.3% respectively. In contrast, 'Eurotonda', 'Seresta' and 'Amanda' had R_s of 1% or less. Overall, the P_f of 'Oberlangen' was higher in all the cultivars tested. In some cultivars P_f surpassed that of 'Chavornay' over twenty fold (Table 4.1).

Table 4.1 Effect of selected potato cultivars on the number of cysts per pot and percentage relative susceptibility (R_s) 12 weeks after inoculation with 5 eggs and second-stage juveniles of *Globodera pallida* populations 'Oberlangen' and 'Chavornay' per ml soil.

	'Oberlangen'		**'Chavornay'**		
Cultivars	**No. cysts[a]**	R_s	**No. cysts[a]**	R_s	**Ratio[b]**
'Désirée'	1,298.5 ± 111.3 a	100	1,158.6 ± 51.6 a	100	1.1
'Albatros'	896.3 ± 39.3 b	69.0	732.6 ± 77.6 b	63.2	1.2
'Laura'	822.4 ± 70.9 b	63.3	617.8 ± 65.3 b	53.3	1.3
'Belana'	590.3 ± 60.1 c	45.5	452.5 ± 32.8 e	39.1	1.3
'Amado'	382.1 ± 22.7 d	29.4	39.3 ± 4.8 d	3.4	9.7
'Ribera'	308.3 ± 25.3 e	23.7	176.3 ± 16.8 f	15.2	1.8
'Euroviva'	277.3 ± 17.8 ef	21.4	16.1 ± 2.7 fg	1.4	17.2
'Eurotonda'	238.6 ± 20.8 f	18.4	11.9 ± 2.7 fh	1.0	20.1
'Seresta'	137.6 ± 15.4 g	10.6	6.6 ± 1.8 gh	0.6	20.8
'Amanda'	89.3 ± 10.0 g	6.9	4.9 ± 1.2 h	0.4	18.3

[a] Mean number of cysts per pot ± standard error of *Globodera pallida* 'Oberlangen' and 'Chavornay'; means in the same column followed by the same letters are not significantly different ($P \leq 0.05$). [b] Ratio = mean number of 'Oberlangen' cysts/mean number of 'Chavornay' cysts.

The R_f of both *G. pallida* populations differed significantly ($P < 0.01$) among the cultivars tested (Fig. 4.5) 'Oberlangen' and 'Chavornay' did not differ in their reproduction on four potato cultivars '', 'Albatros', 'Laura' and 'Belana' (Fig. 4.5).

However, the reproduction on the remaining six cultivars differed significantly ($P < 0.01$) with 'Oberlangen' leading in reproduction rates. 'Chavornay' had an $R_f < 1$ for the cultivars 'Euroviva', 'Eurotonda', 'Seresta' and 'Amanda'. However, the R_f of 'Oberlangen' on these cultivars ranged between 3 and 13 which were significantly higher compared to 'Chavornay' (Fig. 4.5). Overall, 'Oberlangen' had higher R_f than 'Chavornay' in all the cultivars tested.

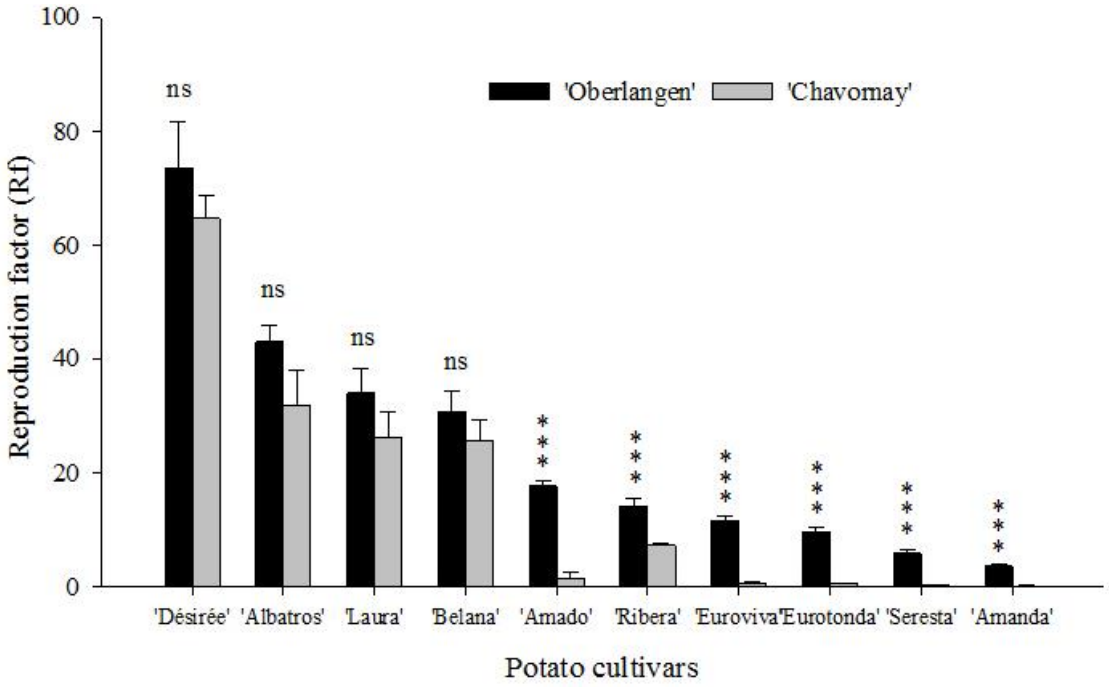

Figure 4.5 Mean reproduction factor ± SE of *Globodera pallida* 'Oberlangen' and 'Chavornay' on ten potato cultivars with different levels of resistance (n = 10). The standard error bars with *ns* were not significantly different at $P \leq 0.05$).

4.4.4 Estimating the Size of the Cysts and the Number of Eggs per Cyst

The cysts of 'Oberlangen' and 'Chavornay' obtained from the susceptible cv. 'Désirée' did not differ in size ($P > 0.05$). However, 'Chavornay' had bigger cysts ($P < 0.05$) on the susceptible cv. when compared with 'Oberlangen' cysts reproduced on the resistant cv. (Table 4.2). The 'Oberlangen' cysts from the susceptible and resistant cultivar did not differ in the size ($P > 0.05$). There was no difference in the numbers of eggs per cyst of the two populations reared on the susceptible cultivar ($P > 0.05$). However, 'Oberlangen' cysts reared on the resistant cultivar had fewer eggs ($P < 0.05$) compared with 'Chavornay' on susceptible cultivar (Table 4.2). The number of eggs in 'Oberlangen' cysts reared on the susceptible and resistant variety did not differ.

Furthermore, 'Oberlangen' had significantly longer males ($P < 0.01$) on both, susceptible cv. 'Désirée' and resistant cv. 'Seresta' compared with 'Chavornay' males from the resistant cultivar. 'Chavornay' males from the susceptible cv. were slightly shorter than 'Oberlangen' males from the same variety (Table 4.2). The 'Oberlangen' and 'Chavornay' J2s did not differ in length ($P >$ 0. 05) regardless of the variety on which the populations were multiplied on (Table 4.2).

Table 4.2 Life history traits of *Globodera pallida* 'Oberlangen' and 'Chavornay' reproduced on susceptible potato cultivar 'Désirée' and resistant cultivar 'Seresta'.

Treatment	Cyst size (µm)[a]	Eggs/cyst[b]	J2 Size (µm)	Male size (mm)
'Chavornay'-'Désirée'	547.6±5.6a[c]	355.9±20.9a	472.9±4.3	1.18±19.6ab
'Oberlangen'-'Désirée'	535.8±4.9ab	339.1±11.6ab	466.8±2.9	1.23±16.8a
'Oberlangen'-'Seresta'	518.4±5.6b	284.5±18.3b	463.8±3.9	1.21±15.6a
'Chavornay'-'Seresta'	nd	nd	nd	1.14±14.3b
P-value	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

[a]Data are means of 40 individual cysts, males and juveniles per population. [b]Data were obtaining from batches of ten cysts replicated ten times. [c]Values within a column having a common letter are not significantly different at $P \leq 0.05$, nd = no data.

Cysts of six virulent and three avirulent populations obtained from potato fields in Emsland region differed significantly ($P < 0.01$) in diameter. The 'Oberlangen' cysts were of the same size as two of the six virulent populations examined (NI-Gpa-VIR004 & NI-Gpa-VIR013). 'Oberlangen' cysts were significantly smaller ($P < 0.01$) than the rest of the virulent populations (NI-Gpa-VIR002, NI-Gpa-VIR003, NI-Gpa-VIR011 & NI-Gpa-VIR012), but larger than the three avirulent populations ((NI-Gpa-AVI001, NI-Gpa-AVI002 & NI-Gpa-AVI003), Fig. 4.6).

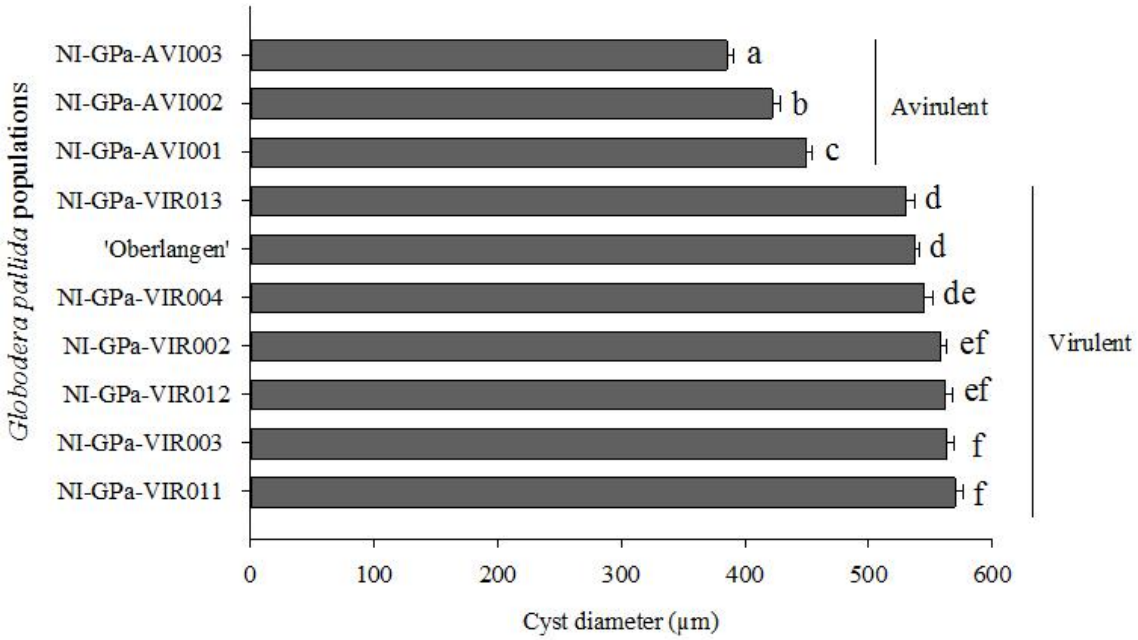

Figure 4.6 Means cyst diameter ± SE of avirulent and virulent populations of *Globodera pallida* ($n = 150$). Standard error bars with a common letter are not significantly different at $P \leq 0.05$.

4.5 Discussion

The *Globodera pallida* populations 'Oberlangen' and 'Chavornay' differed in their development and virulence as well as the life history traits assessed in this study. 'Chavornay' had a slightly higher hatching percentage compared to 'Oberlangen'. 'Oberlangen' had a normal hatching behaviour that cannot account for the increased virulence reported by Niere et al. (2014). Higher hatching of the populations in PRD compared to $ZnCl_2$ was expected. Naturally, the life cycle of *G. pallida* is perfectly synchronized with that of the host (Perry, 1998) and they require root exudates to initiate hatching (Moens et al., 2018). Although *G. pallida* responds better to root exudates compared to *G. rostochiensis* (Den Nijs & Lock, 1992), the latter is able to hatch within the first few days. Conversely, *G. pallida* hatches over several weeks as was observed in this study.

The development of 'Oberlangen' and 'Chavornay' in the roots of the susceptible and resistant potato cultivars showed considerable variations that may account for the differences in virulence between the two nematode populations. The host resistance gene did not prevent the penetration of the juveniles. The number of J2s recovered from the roots of the resistant and susceptible cultivars, 7 DPI, was statistically similar. A higher ratio of males to females was observed in the resistant cultivar compared to the susceptible one. The resistance response of 'Seresta' restricted the development of the syncytium thus depriving the nematode food needed to develop into a female (Schouten, 1993; Bakker et al., 2006; Moen et al., 2018). In this case, the resistance genes were efficient in restricting the development of 'Chavornay', resulting in only few cysts. However, the resistance genes only imposed a partial effect on the 'Oberlangen' population, evident by the number of 'Oberlangen' juveniles developing into females.

To survive in the roots of a resistant host, virulent juveniles evade or suppress the host immune response (Wondafrash et al., 2013). Such juveniles arise following a single mutation in an avirulence gene that enables them to avoid recognition by the host resistance genes. They are able to successfully induce the formation of feeding cells upon which their survival depends (Rice et al., 1985; Bakker et al., 2006). The avirulent juveniles die or moult into males that do not require further nourishment to survive (Trudgill, 1967). The recovery of small sized males of the 'Chavornay' population on the resistant cultivar confirms that they were malnourished in the resistant cultivar. The ability of a section of 'Oberlangen' juveniles to complete their life cycle in the roots of the resistant cultivar confirms the presence of virulent individuals within the

population that are able to circumvent the host resistance (Schouten, 1993). The number of females of the two nematode populations formed on the roots of the susceptible cultivar did not differ. This was expected since the host lacks resistance to PCN and invading juveniles were able to establish themselves and complete the life cycle.

The reproduction of *Globodera pallida* 'Oberlangen' on five commercial cultivars considered resistant to *G. pallida* Pa2/3 was tenfold higher than the reproduction of the reference population 'Chavornay'. The five cultivars 'Amado', 'Euroviva', 'Eurotonda', 'Seresta' and 'Amanda' are rated as resistant to Pa3 where 'Chavornay' was used as the reference population (Anonymous, 2017). However, 'Oberlangen' has adapted to the resistance genes present in the five cultivars and is able to reproduce on them. The five cultivars were therefore considered susceptible to the 'Oberlangen' population. Increased virulence on potatoes carrying quantitative resistance genes is mainly attributed to selection pressure the cultivars impose on nematode population (Turner & Fleming, 2002). This has been demonstrated in various studies (Turner, 1990; Beniers et al., 1995; Schouten & Beniers, 1997; Beniers et al., 2019). The field populations of nematodes have a proportion of virulent alleles inherited from the original introduction (Bakker et al., 2006). Selection pressure imposed on these populations allows the proliferation of virulence alleles leading to emergence of resistance breaking populations.

When the number of eggs per cyst from the susceptible cultivar was estimated, no significant differences were noted between the two populations. The difference in fitness recorded between the two populations was therefore due to the high number of 'Oberlangen' cysts recovered from a susceptible cultivar rather that increased number of eggs per cyst. In their work, Schouten & Beniers (1997) attributed increased virulence of their *G. pallida* population to a high number of J2s able to develop into females, not the increase in the number of eggs per cyst.

The 'Oberlangen' population showed higher virulence and fitness despite having been maintained on a susceptible host since it was reported (Niere et al., 2014). This indicates that the acquired virulence is not reversible as suggested by Castagnone-Sereno et al. (2007) for root-knot nematodes. The stable virulence level of 'Oberlangen' supports the assertion by Turner (1990) that selected PCN populations are stable and distinct from their original population. Indeed, such populations are known to have increased fitness on susceptible cultivars (Fournet et al., 2013) as well as increased cross virulence on other resistant cultivars (Beniers et al., 2019). However, that

could not be confirmed in this study since the source of resistance of some of the cultivars used in this study is not clear.

Differences in cyst content between cultivars differing in their level of resistance has been reported (da Conceiçao et al., 2005). Populations selected on one cultivar do not necessarily have the same level of virulence and fecundity on another host with the same quantitative resistance gene (da Conceiçao et al., 2005). This was demonstrated in our experiment where reproduction of 'Oberlangen' on cultivars with different levels of resistance differed significantly. On the other hand, 'Chavornay' had significantly smaller males on the resistant cultivar compared to males of the 'Oberlangen' population extracted from resistant and susceptible cultivars. Production of the small sized 'Chavornay' males validate the argument that they arose from juveniles that were unable to feed well leading to stunting. However, the size of juveniles of the two populations reared on both potato cultivars did not differ. The viability of the eggs from cysts reared on resistant host was also not affected by the type of resistance in 'Seresta' (unpubl.data).

The size of 'Oberlangen' cysts was compared with collections of six virulent and three avirulent *G. pallida* populations. Based on the size of the cysts, the virulent populations could be placed into two groups; four populations with statistically bigger cysts and three populations with medium sized cysts. The three avirulent populations had significantly smaller cysts. 'Oberlangen' belonged to the medium category but well within the virulent category. In this case, increase in virulence seems to be characterised by increase in cyst size confirming findings by Fournet et al. (2016). However, additional studies are underway to compare the virulence and other life history traits of these virulent and avirulent populations.

The results comparing the life history traits of 'Oberlangen' and 'Chavornay' on the resistant cultivar cannot be interpreted in the light of the existing literature on fitness cost since the original population from where 'Oberlangen' was selected is not known, nor the resistance gene(s) under which the selection occurred. In addition, a definitive categorization of 'Oberlangen' into one of the pathotype groups as defined by Kort et al. (1977) is difficult. This is because 'Oberlangen' is able to reproduce on potato cultivars that are resistant to Pa2 and Pa3 (Niere et al., 2014). This highlights the shortcomings of the pathotyping system by Kort et al. (1977) which classifies *G. pallida* into three pathotypes based on their reproduction on differential cultivars. Emerging resistance breaking populations do not fit in this pathotyping system. Indeed, the expression of the

virulence of a population relative to the reproduction on susceptible cultivar (Anonymous, 2006) is very effective in identifying virulence breaking populations.

Currently there is no known source of resistance to this new virulence type. Future breeding programs should focus on stacking of several QTLs loci (gene pyramiding) into a single cultivar (Dalton et al., 2013; Rigney et al., 2017). This should create an additive effect that would hopefully prolong the resistance efficacy of the cultivar.

In conclusion, our study has revealed that *G. pallida* 'Oberlangen' and 'Chavornay' differ in their development and virulence on potato cultivars categorized as resistant to pathotype Pa2/3 (Anonymous, 2017). 'Oberlangen' is able to reproduce on potato cultivars with different levels of resistance besides having higher fitness on susceptible cultivars. The virulence and fitness of the population has remained unchanged since 2014 despite the population being permanently maintained on a susceptible cultivar. This confirms the stability of this population and demonstrates lack of tradeoff following selection in the field. Therefore, 'Oberlangen' is a suitable candidate population for use as a reference when testing new potato germplasm and breeding material for resistance against this new virulence type of *Globodera pallida.*

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Chapter 5: Reproduction of *Globodera pallida* on Tissue Culture (TC) Derived Potato Plants and their Potential Use in Resistance Screening Process

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5.1 Abstract

Globodera pallida infestation on potato is responsible for huge yield losses globally. Screening of potato germplasm for resistance to the nematode at the early stages of breeding programmes can significantly enhance resistance-based management. This study assessed the suitability of tissue culture (TC) derived potato plants as screening material for resistance to *G. pallida*. Reproduction of the nematode on TC plants was similar to the reproduction on tubers and eye-plug derived plants. The pot volume, inoculum density and inoculation time had a significant effect on the reproduction. A positive correlation was found between the mean number of white females on the root surface and the final number of nematode cysts after extraction. Resistance ranking using TC plants and the tubers yielded comparable results thus justifying the use of TC in the screening process. Tissue culture plants have the potential of speeding up the screening process and reducing resource requirements, thus lowering breeding cost.

Keywords
Breeding, *Globodera rostochiensis*, management, Potato cyst nematodes

5.2 Introduction

For nearly a century, Potato Cyst Nematodes (PCN) *Globodera rostochiensis* (Woll.) and *Globodera pallida* (Stone) have remained a major constraint in potato production globally (Minnis *et al*., 2004). These pests induce significant yield losses (Alonso *et al*., 2011) and for this reason, they are regulated as quarantine organisms in many countries (Anonymous, 2013). Several management strategies exist, but none is fully effective against these nematodes (Viaene *et al*., 2013). Potato growers have relied on chemical management for decades, but these chemicals have adverse effect to the environment thus raising major concerns (Sheridan *et al*., 2004). The use of resistant potato cultivars is more effective in controlling PCN than pesticide applications (Molinari, 2011).

Resistant potato cultivars have several defence mechanisms that restrain the reproduction of nematode (Sobczak *et al*., 2005). These mechanisms include hypersensitive response where juveniles inside the host roots are surrounded by necrotized cells that prevent them from initiating the formation of feeding sites also known as syncytium (Moens *et al*., 2018). Another mechanism is by restricting the development of syncytium following the degeneration of tissues surrounding

it. This leads to the death of the nematode or tilts the sex ratio in favour of males (Moens *et al*., 2018). The ability of resistant potato plants to restrict the reproduction of nematode provides a key management option. If well implemented, resistance based management can be more sustainable, cost effective and environmental friendly compared to the application of chemicals (Starr *et al*., 2013; Milczarek *et al*., 2014; Davis & Elling, 2015). Therefore, there is need to enhance the use of resistant potato cultivars in the management of *G. pallida*. This can be achieved by supporting breeding programmes through availing methods that promote effective, economical and faster means of screening breeding materials. This would allow selection of candidate clones at the early stages of breeding programme.

The process of breeding potato for resistance to PCN is long, tedious and cost intensive (Castagnone-Sereno, 2002; Molinari, 2011; Gopal, 2015). It involves screening thousands of candidate plant genotypes to select desired traits from a pool of progenies (Gopal, 2015; Mori *et al*., 2015) a process that is sometimes hampered by limited resources (Plaisted, 1984). A method of screening resistant materials at the early stages of breeding programme would significantly enhance the process (Plaisted *et al*., 1984) and ensure optimal utilization of the resistant potatoes in PCN management. Such a system should be efficient, reliable, accurate and consistent over time and space (Cook & Noel., 2002; Molinari, 2011; Starr *et al*., 2013; Milczarek *et al*., 2014).

Often, potato tubers are used as screening material in breeding programmes (Forrest & Holliday 1979; Finlay *et al*., 1998; Anonymous, 2006). However, tubers tend to delay the screening process as it takes time to generate micro-tubers from *in vitro* breeding lines and subsequently multiply them to have adequate numbers for screening (Phillips, 1981; Gopal, 2015). Furthermore, resistance tests with tubers are resource and labour intensive (Phillip, 1981) because they require use of larger pots and occupy large area of greenhouse space. Several other screening methods have been used in the past, they include: bulky sowing of seedlings (Phillips, 1981) although seedlings are not genetically identical and therefore not suitable for testing clones (George e*t al*., 2008). Screening tests using closed containers (Foot, 1977; Phillips *et al*., 1980) require the use of tubers thus prolonging the waiting duration. I*n vitro* screening which is a laboratory based method where materials are tested on artificial media (Mugniéry, 1989; Janseen *et al*., 1990; Arntzen *et al*., 1994; Fournet *et al*., 2016). Although the use of artificial media is a rapid way of screening material and requires use of a small space (Franks *et al*., 2003), the method is not suitable when

screening thousands of genotypes because of the challenge of media contamination. In addition, the method potentially underestimates the degree of resistance (Fleming, 1998). Plants derived from potato stem cuttings have also been used in resistance screening successfully (Arntzen & van Eeuwijk, 1992). However use of tissue culture (TC) derived potato plants as screening material has not been explored.

Tissue culture plants are generated using micro-propagation technique that allows for the production of genetically identical clones from a common mother plant (Murashige, 1974). Furthermore, the clones are free of seed borne diseases; they are uniform in size and genetically similar. These characteristics make TC plants suitable candidates for assessing the resistance of potato germplasm to PCN (George *et al*., 2008). Use of TC plants eliminates the need for micro tuber propagation, a process that takes time and hence delays the breeding process (Gopal, 2015). This study was done to assess the suitability of TC derived potato plants in comparison to plants derived from tubers and eye-pug for use when screening material for resistance against *G. pallida*. Experiments were carried out to: (i) compare the relationship between the number of white females of *G. pallida* counted on the root surface and the final number of cysts after sample extraction, ii) assess the effect of planting materials on nematode reproduction, iii) evaluate the effect of pot volume on nematode reproduction, (iv) assess the influence of inoculum density on nematode reproduction, (v) assess the effect of inoculation timing on nematode reproduction and, (vi) compare the resistance rating of selected potato cultivars using TC derived potato plants and tubers following the standard EPPO protocol.

5.3 Materials and Methods

5.3.1 Plant Material

In vitro propagated TC potato plants and potato tubers were used in this study. Five different potato cultivars were used in different experiments, they included; ‘Amado’, ‘Belana’, ‘Désirée’, ‘Eurotonda’ and ‘Ribera’. Tissue culture plantlets, approximately 30 mm tall, were carefully removed from propagation tubes. The culture medium adhering to the root system was gently washed off using running tap water prior to planting. Only small sized tubers (approx. 15 g) or eye-plugs were used. The tubers were germinated in darkness at room temperature. Upon shooting, they were transferred into a well lit room for shoot hardening.

Growth substrate

Loess soil (Müller and Rumpenhorst, 2000) was used as substrate. The soil was enriched with slow release fertiliser ((Osmocote Exact Standard® Everris International B.v, Netherlands) 15% N, 9% P2O5, 12% K2O and 2% MgO) at a rate of 1.5 g per kg soil^{-1}.

Inoculum preparation

The reference population *G. pallida* (Pa2/3) 'Chavornay' was used in this study. The population was multiplied and maintained on the susceptible potato cv. 'Désirée' in the glasshouse and cysts subsequently stored at 4 ºC for a minimum period of six months to overcome diapause (Perry *et al*., 2013) before being used in the experiments. Inoculum was prepared by soaking cysts in tap water overnight at room temperature. The soaked cysts were then crushed to free eggs and juveniles (Seinhorst and Den-Ouden, 1966). The suspension was adjusted to achieve an estimated number of 500 eggs and J2 per ml^{-1}.

Glasshouse conditions

Experiments were conducted under controlled conditions in a glasshouse using a long-day light regime (16 hrs supplemental light). The relative humidity was maintained at a range of 50-70%. The glasshouse had an automated system for cooling during summer and heating during winter such that temperatures were maintained at around 18 ± 2 ºC throughout the experiments. A Testo® 175T3 (Testo Ltd, UK) temperature logger was used to monitor and record the glasshouse temperature at hourly intervals.

Planting

During the planting of TC plants, pots were filled with the 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 the tubers and 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. 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. Each experiment was conducted twice.

Sample processing and data collection

Nematode cysts were extracted by washing the entire soil through a 250 µm sieve. The cysts and debris on the sieve were then collected into a funnel containing filter paper. The filter papers were left to dry for a week at room temperature. Cysts were separated from debris by acetone extraction (Seinhorst, 1974). The recovered cysts were counted and recorded. To estimate the cyst content, all the extracted cysts were soaked in water and crushed to free eggs and J2 (Seinhorst and Den-Ouden, 1966). The eggs and J2 were washed into a beaker and water added to create an eggs and J2 suspension. To quantify the number of eggs and J2, the suspension was thoroughly mixed using SCHEGO® air pump and 1ml suspension transferred into a counting slide. Eggs and J2 in 1 ml subsamples were counted using Leitz Labovert® inverted microscope at 40x magnification. Counting was done three times and the mean number of eggs and J2 ml^{-1} water determined (Seinhorst and Den-Ouden, 1966; Van Bezooijen, 2006). The data were used to calculate the number of eggs and J2 per cyst.

5.3.2 Experimental Set Up

Visual assessment of white females on the root surface

The experiment was setup to assess the relationship between the mean number of the white *G. pallida* females visible on the root surface and the actual number of cysts after extraction. Tissue culture plantlets of susceptible cv. 'Désirée' and resistant cv. 'Eurotonda' were planted in 192 cm^3 pots with ten replicates each. The 192 cm^3 pots were small, transparent, plastic pots measuring 4 cm x 4 cm x 12 cm height. These pots allowed visual examination and counting of white nematode females on the root surface through the transparent walls. The pots were placed in a stainless steel metal box in a completely randomized order. Fourteen days after planting, each pot was inoculated with *G. pallida* eggs and J2. 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 P_i of 5 eggs and J2s ml^{-1} soil (Anonymous, 2006). The holes were then carefully covered with soil. Four weeks after inoculation, the presence of white females on the root surface was assessed by removing the pots from the box and counting the number of visible white females. The number of females was recorded and the pot returned into the box for the females to complete the life cycle. Visual counting of females was done weekly until a change of the colour of females was noted. The mean

number of visible white females per pot was determined. Upon the termination of the experiment, cysts were extracted from the soil sample and the final number of cysts per pot determined.

Effect of planting materials on nematode reproduction

The aim of this experiment was to assess whether the nature of planting material affects *G. pallida* reproduction. The tests were done using TC plants, tubers and eye-plugs of the susceptible cv. 'Désirée'. Tubers served as control in this experiment. Each of the three types of planting material was grown in 192 cm^3 and 500 cm^3 pots with five replicates. Fourteen days after planting, each plant was inoculated with 5 eggs and J2 of *G. pallida* (ml soil)$^{-1}$ as described above and randomized on the glasshouse bench.

Effects of pot volume on nematode reproduction

This experiment was set to study the effect of pot volume on the reproduction of *G. pallida* on TC plants. Tissue culture plants of the susceptible cv. 'Désirée' were planted in pots of four different volumes (192, 500, 1000 or 1500 cm^3) with six replications each. Fourteen days after planting, each pot was inoculated with 5 eggs and J2 of *G. pallida* (ml soil)$^{-1}$. Pots were completely randomized and maintained in the glasshouse.

Effects of initial population density (P_i) on nematode reproduction

This experiment was done to determine the effect of initial population density on the reproduction of *G. pallida* on TC plants. Plantlets of the susceptible cv. 'Désirée' were planted in 192 cm^3 pots fitted in the metal box. Fourteen days after planting, six pots per treatment were inoculated with 1, 5, 10, or 20 eggs and J2s ml^{-1} soil and left on a glasshouse bench for the entire duration of the experiment.

Timing of inoculation

To investigate the effect of inoculation time on the reproduction of *G. pallida,* TC plants of the susceptible cv. 'Désirée' and the partially resistant cv. 'Ribera' were planted in 192 cm^3 pots randomized in the metal boxes. Six plants of each cv. were inoculated with 5 eggs and J2 of *G. pallida* (ml soil)$^{-1}$ at 0, 7, 14, 21, 28 and 35 days after planting (dap). Plants in each of the six treatments were left to grow in the glasshouse for 12 weeks after inoculation.

Validation of resistance levels

The aim of this experiment was to test whether resistance tests of selected potato cultivars against *G. pallida* using TC clones and tubers following the EPPO standard resistance testing procedure (Anonymous, 2006) could generate comparable results to justify the use of TC clones in resistance testing. Five potato cultivars with known different levels of resistance to *G. pallida* (Pa2/3) 'Chavornay' population were used in these tests. The cultivars used were; 'Désirée' (resistance score = 1), 'Belana' (susceptible to *G. pallida*), 'Ribera' (resistance score = 6) and 'Amado' (resistant to *G. pallida*) and 'Eurotonda' (resistance score = 9) (*Anonymous*, 2017). The resistance levels of the potato cultivars were evaluated using TC plants in 192 cm^3 and 250 cm^3 pots, with ten replicates per cultivar. The five cultivars were also tested using tubers in 1000 cm^3 pots following the EPPO guidelines (Anonymous, 2006) with each cv. replicated five times. Fourteen days after planting, each pot was inoculated with 5 eggs and J2 of *G. pallida* $(ml\ soil)^{-1}$.

5.3.3 Data Analysis

The number of cysts per plant was multiplied by the average number of eggs and J2s per cyst to estimate the final nematode population (P_f). The P_f was then used to calculate the reproduction factor (R_f) by dividing the final population (P_f) with the initial population (P_i) ($R_f = P_f/P_i$). To compare the resistance rating of the TC plants and tubers following the standard EPPO protocol, the relative susceptibility (R_s) of the test cv. was determined as $Pf_{\text{test cultivar}}/Pf_{\text{standard susceptible control cultivar}} \times 100\%$ where cv. 'Désirée' was used as the standard susceptible control. The level of resistance was determined using the EPPO score scale as follows:- 1 (>100), 2 (50.1–100%), 3 (25.1–50%), 4 (15.1–25%), 5 (10.1–15%), 6 (5.1–10%), 7 (3.1–5%), 8 (1.1–3%), 9 (≤1%). Where 9 represent the highest level of resistance and 1 represent the lowest resistance level (Anonymous, 2006). There were no significant differences ($P > 0.05$) between experiments testing how planting material and pot volume affect the multiplication of *G. pallida,* as well as the experiments to validate the resistance level of selected potato cultivars. Therefore, data from two experiments were pooled and analysed for variance using R-software version 3.5.0 (R Foundation for Statistical Computing). Dunnett's test was used to compare means of TC and eye-plugs that were different from the control (tuber) while Tukey's HSD test was used to compare statistical means in the experiment testing the effect of pot volume and in the resistance validation experiment. Means were considered different at $P < 0.05$ level of significance. The relationship between the number of white females on the root surface and the final number of cysts per pot after extraction was

assessed using correlation analysis. A quadratic regression analysis was performed on the final population density (P_f) and reproduction factor (R_f) as functions of initial population density (P_i) and inoculation time. Regression analyses were done using means of two experiments. The analyses were performed using Sigma plot 13.0 (SYSstat Software Inc., Chicago).

5.4 Results

5.4.1 Visual Counting of White Females

When the mean number of white females on the root surface of susceptible cv. 'Désirée' was correlated with the number of cysts per pot, a significant positive relationship ($P = 0.0001$; $R^2 = 0.428$) was found (Fig. 5.1).

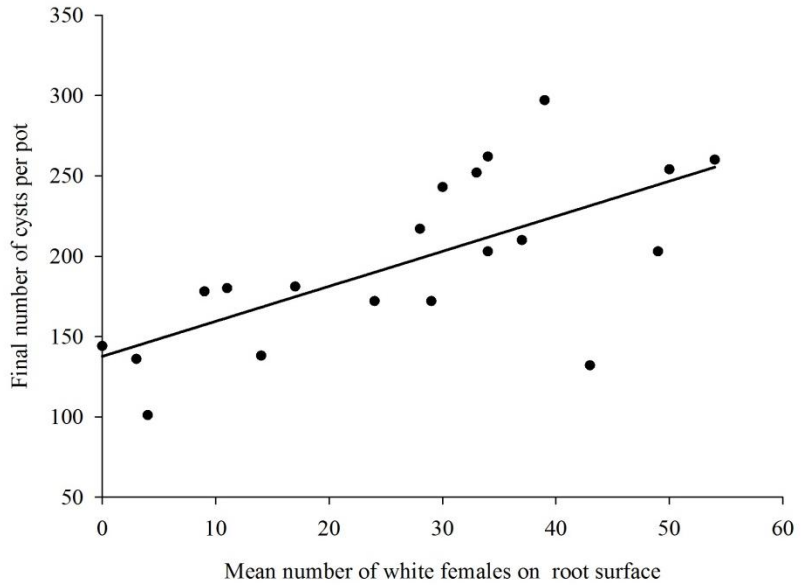

Figure 5.1 Correlation between the mean numbers of white female of *G. pallida* counted on the root surface of the susceptible TC potato cultivar cv. 'Désirée' and the final number of cysts per pot after extraction. Each data point is a mean of four counts. The linear equation based on the mean is y = 2.18x + 137.62; R2 = 0.428; $P < 0.0001$; $n = 20$

However, the correlation between the mean number of white females on the root surface of a resistant cv. 'Eurotonda' and the final number of cysts could not be analysed since most of pots had no visible cysts on the root surface and only a few cysts after extraction. In general, an average of 0.3 ('Eurotonda') and 19 ('Désirée') white females were counted on the root surfaces compared to 2.0 ('Eurotonda') and 128 ('Désirée') cysts that were extracted at the end of the experiments.

Therefore, the number of visible females on the root surface represented approximately 10-13 % of the total number of cysts after extraction.

5.4.2 Effect of Planting Material

The multiplication of *G. pallida* on TC derived plants was not different from the multiplication on the tuber derived plants in 192 cm^3 pots. Nematode reproduction on eye-plug derived plants was significantly ($P < 0.05$) higher with an R_f of 51.15 compared with the tubers and TC plants which had an R_f of 39.89 and 31.38, respectively (Fig. 5.2-A). Reproduction of *G. pallida* on TC plants grown in 500 cm^3 pot was significantly lower ($P > 0.05$) with R_f of 16.77 compared with eye-plugs and tubers with a R_f of 23.30 and 29.67, respectively (Fig. 5.2-B).

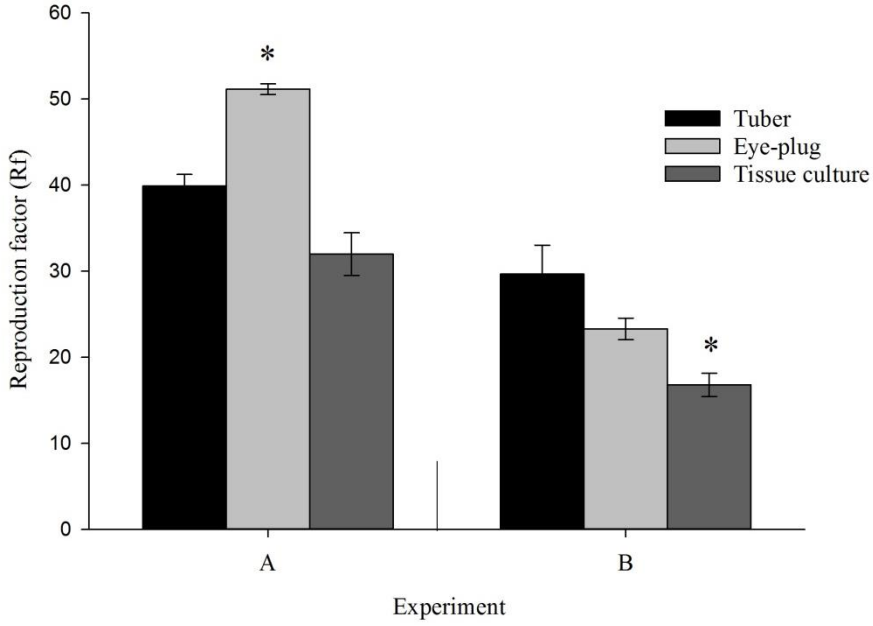

Figure 5.2 Mean reproduction factor (± SE) of *G. pallida* on tuber, eye-plug and tissue culture derived potato cultivar cv. 'Désirée'. A: In 192 cm^3 and B: 500 cm^3 pot volume. * indicates significant difference ($P < 0.05$) from the control (Tuber) by Dunnett's test. Vertical bars are standard error of the means (n = 10).

5.4.3 Effects of Pot Volume

The volume of the pots affected the multiplication rate and thus the final nematode population density. The number of nematodes per pot increased with the increase in pot volume while the

reproduction factor decreased with the larger pot volumes. Smaller pots (192 cm^3) had a significantly higher ($P < 0.05$) reproduction (R_f = 32.55) while 1500 cm^3 pot had a significantly lower reproduction (R_f = 10.09, compared to 500 cm^3 (R_f = 22.35) and 1000 cm^3 (R_f = 15.13) pot volumes (Fig. 5.3). On the other hand, high numbers ($P < 0.05$) of nematode eggs and J2s were recorded in 1500 cm^3 pots compared to 1000, 500 and 192 cm^3 pots which showed lower number of eggs and J2s depending on the pot volume (unpublished data).

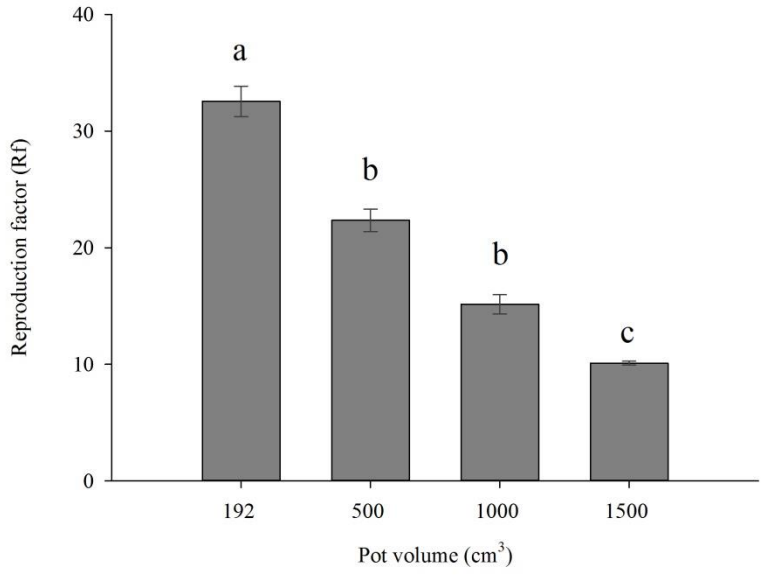

Figure 5.3 Mean reproduction factor (± SE) of *G. pallida* on susceptible TC potato, cultivar 'Désirée', in different pot volumes. Means with the same letters are not statistically different ($P > 0.05$) according to Tukey's HSD test. Vertical bars are standard error of the means (n = 12).

5.4.4 Effects of Initial Population Density

The final population density of *G. pallida* on cv. 'Désirée' was positively correlated with the inoculum density ($P < 0.0048$; R^2= 0.955). The P_f increased with the increase in P_i to reach the highest level of 186 eggs and J2 ml^{-1} soil at the P_i of 20 eggs and J2s per ml^{-1} (Fig. 5.4). On the other hand, the reproduction factor was negatively affected ($P < 0.0001$; R^2 = 0.926) with the increase of P_i (unpublished data). Plants inoculated with 1 egg and J2 per ml^{-1} soil recorded a higher R_f (72.87) while plants inoculated with 20 eggs and J2 ml^{-1} soil recorded the lowest (R_f = 10.92).

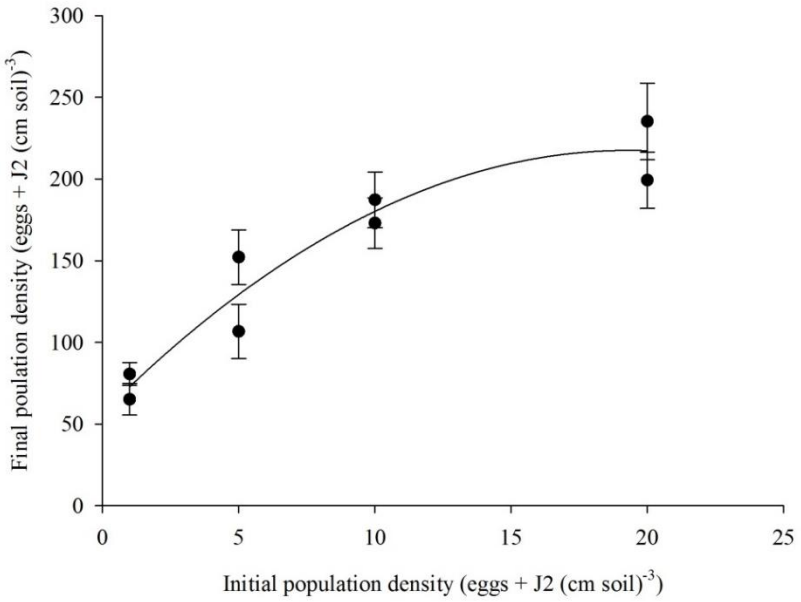

Figure 5.4 Relationship between the initial population (P_i) and final population density (P_f) of *G. pallida* on the TC plant cv. 'Désirée'. Data are from two experiments, each data point is a mean of six replicates ± SE. The polynomial quadratic model based on the mean is $y = -0.43x^2+16.70x+56.60$; $R^2 = 0.926$; $P = 0.0165$. J2 = second-stage juveniles.

5.4.5 Timing of Inoculation

The reproduction factor (R_f) of *G. pallida* on the susceptible cv. 'Désirée' showed a negative relationship with time of inoculation ($P < 0.0001$; $R^2 = 0.898$, Fig. 5.5). Similarly, the P_f on the susceptible cultivar decreased ($P < 0.0001$; $R^2 = 0.870$) with late inoculation (unpublished data). A similar effect was found for the partially resistant cv. 'Ribera'. There was a significant negative relationship ($P < 0.0001$; $R^2 = 0.945$) between the inoculation time and the R_f (Fig. 5.6) on one hand and between the inoculation time and P_f ($P < 0.0001$; $R^2 = 905$) on the other hand (unpublished data).

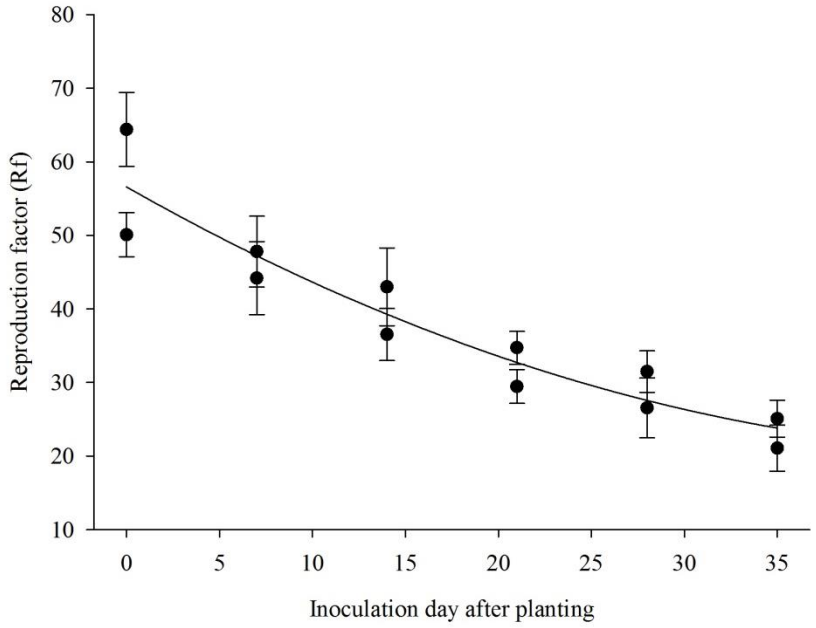

Figure 5.5 Relationship between the reproduction factors (R_f) of *G. pallida* on TC plants of the susceptible cv. 'Désirée' and the time of inoculation in days after planting (DAP). Data are from two experiments, each data point is a mean of six replicates ± SE. The polynomial quadratic model based on the mean in $y = 0.014x^2 – 1.436x + 56.81$; $R^2 = 0.898$; $P < 0.0001$.

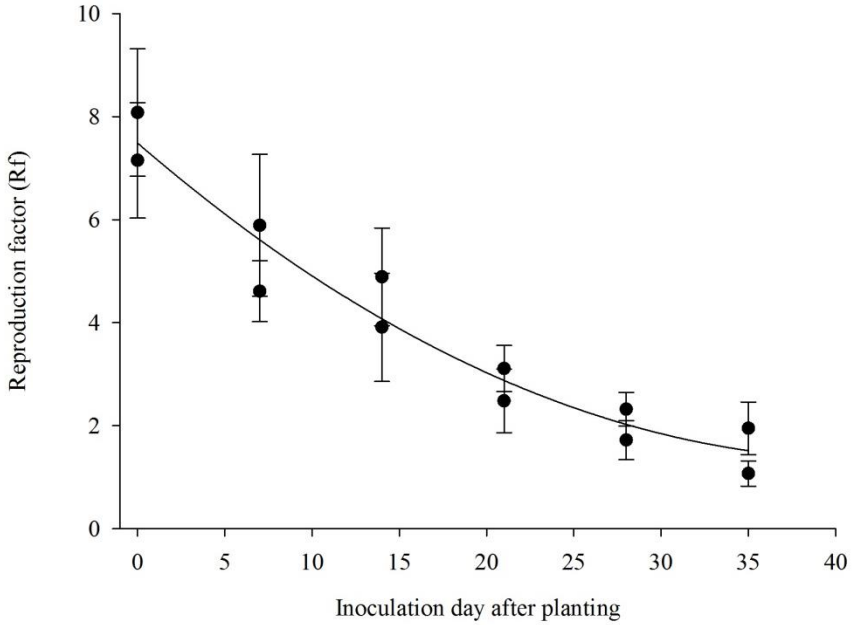

Figure 5.6 Relationship between the reproduction factors (R_f) of *G. pallida* on TC plants of the partially resistant cv. 'Ribera' and the time of inoculation in days after planting (DAP). Data are from two experiments, each data point is a mean of six replicates ± SE. The polynomial quadratic model based on the mean is $y = 0.004x2 – 0.293x + 7.489$; $R^2 = 0.945$; $P < 0.0001$

5.4.6 Validation of Resistance Classes

The relative susceptibility and resistance ranking of five cultivars assessed as TC plants in 192 cm^3 and 250 cm^3 pot volumes were similar. The relative susceptibility (R_s) and resistance ranking (R_l) of the TC plants matched the data obtained with tuber derived plants following the EPPO standard protocol (Table 5.1). The resistance ranking of the cultivars was similar for all the three tests except one case where the TC system rated the cv. 'Eurotonda' a class lower compared to the standard test. Higher reproduction factors were observed with the standard test compared to TC plants for all the five cultivars tested. However, no difference was observed concerning the relative susceptibility of the cultivars.

Table 5.1 Resistance rating of five cultivars assessed with TC derived potato plants (in 192 cm^3 and 250 cm^3 pots) and potato tuber (as per the EPPO standard protocol)†.

Cultivar	TC plants (192 cm^3)			TC plants (250 cm^3)			Tubers (1000 cm^3)		
	R_f ± S.E	R_s (%)	R_l	R_f ± S.E	R_s (%)	R_l	R_f ± S.E	R_s (%)	R_l
'Désirée'	33.18±5.31a‡	100	1	32.68±8.85a	100	1	61.75±5.92a	100	1
'Belana'	9.61±2.02b	29.03	3	9.94±2.22b	30.43	3	19.43±2.06b	31.46	3
'Ribera'	5.77±1.04c	17.45	4	5.72±1.39c	17.52	4	10.33±1.49c	16.73	4
'Amado'	0.87±0.28d	2.63	8	0.47±0.17d	1.44	8	1.21±0.10d	1.96	8
'Eurotonda'	0.37±0.11d	1.12	8	0.32±0.12d	1	8	0.32±0.05	0.52	9

†The table shows the mean reproduction factor (R_f) ± standard error (S.E), the relative susceptibility (R_s) and the respective resistance level (R_l) of the five potato cultivars.
‡ Means within columns with different lower case letters are significantly different ($P < 0.05$; TC plants $n = 20$; Tubers $n = 10$).

5.5 Discussion

Assessment of potato germplasm for resistance to PCN is an important component of breeding programmes. There are several methods of assessing the resistance mainly using potato tubers. Use of *in vitro* derived plants as screening material for resistance to PCN had not been explored before and therefore this study examined the possibility of utilizing the TC material by studying the reproduction of *G. pallida* on TC plants and factors affecting the reproduction.

Extraction of cysts from soil samples is tedious and time consuming. Counting the white females on the root surface could be a quick alternative to the extraction. Tests were conducted to determine if a relationship existed between the mean number of white females on the root surface of the susceptible cv. 'Désirée' and resistant cv. 'Eurotonda', and the actual number of cysts after extraction. The number of white females on the root surface was relatively low or absent on some

pots examined. The inability to detect white females on the roots of the resistant potato cv. despite having cysts in the soil, and the presence of very few cysts on the root surface of a susceptible cv. shows the difficulty of using white female counts in determining the level of resistance of a cultivar. Counting of white females on the root surface has been used with tuber derived potatoes (Phillips *et al*., 1980; Plaisted, 1984 & Faggian *et al*., 2012). However, the suitability of the method on assessing TC derived potatoes had not been investigated. While using visual assessment, Plaisted *et al* (1984) recommended that plants with 0-5 cysts on the root surface are considered resistant while those with more than five cysts susceptible. Faggian *et al*., (2012) classified potatoes with three or more visible females as susceptible while those with no visible female were considered resistant, as long as the results were consistent for all the replicates. However, it was not possible to classify partially resistant cultivars based on visual counts of female nematodes on the root ball surface (Faggian *et al*., 2012). This study confirms previous observations that counting the number of white females on the root surface may not be sufficient to measure the resistance level of TC derived plants to the accuracy required to assess resistance on the 1-9 scale (Anonymous, 2006). Counting the white females can be challenging especially when the cysts are very small in size (Gonzalez *et al*., 1996) and almost undetectable by visual examination. In addition, the *G. pallida* females mature at different times and the colour changes from white to brown upon maturity. Therefore, visual detection is only possible within a short period of time (Anonymous, 2013). However, counting white females on the root surface could be used to screen and eliminate cultivars with low levels of resistance so that assessment that is more detailed can be done on cultivars that are more resistant. In such a case, plants with visible cyst(s) should be regarded as susceptible and therefore discarded while physical extraction of cysts should be done on pots without few or no visible females (Turner, 1989).

When testing potato cultivar to assess the resistance to PCN, an R_f (P_f/P_i) of over x20 should be achieved on the susceptible control variety for it to be used as a reference susceptible control (Anonymous, 2006). In this study, the multiplication rate of *G. pallida* on TC plant cv. 'Désirée' surpassed the above required reproduction rate with R_f of 31.38. This confirms the suitability of TC plants for assessing plant resistance. The multiplication on TC plants grown in 192 cm^3 pots was similar to multiplication on the tuber derived plants. In 500 cm^3 pots, the multiplication of *G. pallida* on TC plant was slightly lower, but still within the range recommended by McKenzie & Turner (1987). As confirmed in this study, TC plants are comparable to tubers and eye-plugs when

screening potatoes for resistance against *G. pallida*, because they allow high nematode multiplication rates.

Pot sizes are known to influence the reproduction and the final population density of nematodes on tuber derived experiments (McKenzie & Turner, 1987). This study confirms the above mentioned observations. Using TC plants, the pot volume had a significant effect on nematode multiplication. The P_f increased with the increase in the pot volume while the R_f decreased with increase in volume. Larger pots allow better plant growth by providing adequate space for root formation and consequently support high numbers of nematodes (McKenzie & Turner, 1987). A positive relationship between the pot volume, and shoot and root dry weight of test plants was observed in this study (unpublished data). As such, one would expect an increase in R_f with the increase in pot size (bigger plant size). However, R_f is density dependent, it varies depending on the P_i and the plant root densities (Gill & Swarup, 1979). In our study, the R_f decreased with the increase in pot volume because the P_i was calculated per volume of soil (Anonymous 2006) and plants in larger pots received a higher number of nematodes compared to plants in smaller pots. The reduction in R_f with the increase in pot volume can therefore be attributed to the competition among nematodes due to the high number of nematodes inoculated (Castillo *et al*., 1995). A direct positive relationship between R_f and pot size (or plant size) could be realized if plants in different pot volumes were inoculated with same number of nematodes.

The multiplication rate of *G. pallida* on TC plants was negatively correlated with the P_i. However, the P_f increased with increase in P_i. This effect has been reported on tuber and eye-plug potatoes (Phillips, 1984; Seinhorst, 1984; Lamondia & Brodie, 1986; Brodie and Brucato, 1993), but was never been assessed for TC plants. A number of factors may account for the decline in reproduction with increased P_i. Firstly, increase in P_i induces competition between individual nematodes (Wallace, 1969; Evans, 1993; Castillo *et al*., 1995), food deprived juveniles moult to males (Pariyar *et al*., 2010) or they fail to develop and therefore die (McClure and Viglierchio, 1966; Cook & Noel, 2002). Secondly, high numbers of nematodes damage the root system and inhibit root growth (Wallace, 1966; Greco & Di vico, 2009; Schomaker & Been, 2013) affecting plant development and consequently lowering nematode reproduction. At a higher P_i, reproduction starts to decrease until P_f falls below the P_i level (Evans, 1993). However, the maximum P_i used in this study (20 eggs and J2 ml^{-1} soil) was not high enough to push the P_f below P_i. Several studies

have recommended using lower P_i levels (Seinhorst, 1984; Lamondia and Brodie, 1986), because it allows nematodes to express their virulence potential (Mugniéry *et al*., 1989) without having a detrimental effect on the host growth (McKenzie & Turner, 1987). In this study, the recommended P_i of 5 eggs and J2 ml^{-1} soil (Anonymous, 2006) proved to be optimal for testing TC plants.

The time of inoculation had a clear effect on nematode reproduction, lowering both the R_f and P_f on susceptible and partially resistant cultivars. Highest multiplication rates were achieved with inoculation soon after planting (Fig. 5.5 & 5.6), confirming the report by Rao & Peachy (1966) that delayed inoculation results in lower reproduction and consequently less effect on plant height and yield. In most tests with cysts used as inoculum, inoculation is done before planting by either mixing the cysts with the substrate (Greco *et al*., 2005) or by packing cysts in nylon sachets (McKenzie & Turner, 1989) and burying the sachets in the soil. Inoculation with eggs (as was the case in this study) or hatched juveniles is done several days after germination (Turner and Stone, 1984). The type of inoculum used determines the rate of juvenile penetration (Von Mende, 1997). Where hatched juveniles are used, penetration begins within hours. However, when cysts or eggs are used, a number of physiological and chemical processes take place culminating in hatching (Perry *et al*., 2013) followed by penetration. There is a time lag between inoculation and penetration and according to the results presented, early inoculation seems to favour hatching and penetration and consequently leads to higher reproduction. Cook and Noel (2002) suggest delaying of inoculation to allow root formation as a way of reducing competition among nematodes. However, according to our study, early exposure of TC plants to *G. pallida* is important in order to have a higher multiplication rate, which is necessary in assessment of resistance. Therefore, inoculation of TC plants should be performed within seven days after planting.

Tests done with TC plants and the tubers following the EPPO protocol generated comparable resistance rating. The ranking of cv. ‘Eurotonda’ as the most resistant and ‘Désirée’ as the most susceptible cv. was in agreement with the official published cultivar descriptions (*Anonymous*, 2017). Using TC plants, it was possible to discriminate susceptible, partially, and fully resistant plants making the tests suitable for screening breeding materials possessing monogenic or polygenic resistance (Trudgill, 1991). Although the P_f and R_f of *G. pallida* were different in the three tests, the relative susceptibility (R_s) and resistance level (R_l) of the cultivars did not vary significantly. Variation in P_f and R_f in different test systems is common (Cook & Noel, 2002), but

it does not necessarily change resistance ranking assessed in relation to a standard susceptible cultivar (Mugniéry *et al*., 1989; Phillips *et al*., 1989; Cook & Noel, 2002). The difference in P_f and R_f are due to the interaction between host genetic composition and the host-environment (Phillips *et al.,* 1989; Trudgill, 1991) making reproduction vary with time and place (Arntzen & Eeuwijk, 1992).

In this study, the suitability of TC plants as screening material for resistance against *G. pallida* in potato plants was assessed. The plants were shown to allow multiplication of nematodes to level that is suitable for resistance screening. The ability to determine the resistance level of TC plants in small pot volumes significantly reduces the amount of resources in the process such as substrate, glasshouse space and personnel. This should prove useful to the breeders because TC derived plants can speed up the screening process and significantly lower the costs associated with the breeding programme.

5.6 Acknowledgements

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Chapter 6: General Discussion

This study aimed at characterizing the golden potato cyst nematodes (PCN), *Globodera rostochiensis,* from Kenya in glasshouse and laboratory experiments. Their biology, pathotype, and diapause requirements were investigated in Chapter 2 while the genetic diversity and population structure were investigated in Chapter 3. In addition, a resistance breaking population of *G. pallida, '*Oberlangen', was characterised by assessing the life history traits associated with increased virulence (Chapter 4) with the aim of identifying traits associated with increased virulence. Finally, in order to enhance the breeding for PCN resistance, the suitability of tissue culture potato plants as screening material for resistance to PCN was demonstrated and established (Chapter 5).

6.1 The Characteristics of *G. rostochiensis* Populations from Kenyan

Resistance to potato cyst nematodes is species and pathotype specific (Bakker et al., 2006). To effectively utilize the available resistance genes, information regarding the target populations is therefore essential (da Cunha., 2012). The six Kenyan populations assessed alongside the German population 'Ecosse' were not able to reproduce on cultivars carrying the *H1* gene. Most of the cultivars tested carry this gene and thus were resistant to the Kenyan populations (Chapter 2). The performance of the tested cultivars could be extrapolated in the management of PCN in Kenya. The *H1* gene is stable and it is not significantly influenced by the genotype-environment interactions (Phillips, 1985; Mugniéry et al., 1989) making is suitable for nematode management in different environmental conditions. Nevertheless, variations in aggressiveness were recorded among the Kenyan populations indicating that there is potential for selection for increased virulence over time (Chapter 2). Also, most importantly, the Kenyan populations have no obligatory diapause unlike other PCN populations from temperate areas of the world (Moens et al., 2018). Indeed, multiple generations were achieved in less than a year without diapause. This is the first documented lack of obligatory diapause following continued inoculation of newly formed cysts on a new host plant of the same species. The experiments were designed to simulate the potato cropping systems in Kenya where farmers harvest potatoes and plant the next immediately in order to achieve two to three crop cycles per year (Muthoni et al., 2013). The intensive potato production, with minimum or no crop rotation (Muthoni et al., 2013) allows the build-up of nematode populations within a short period since nematodes are able to achieve multiple generations in a year (see Chapter 2). There are reports of PCN populations that are able

to complete more than one generation on the same host (Philis, 1980; Greco et al., 1988; Jimenez-Perez et at., 2009; Mimee et al., 2015). However, the findings in this study were different because new generations were achieved on a new host plant representing a new crop cycle.

The development of *G. rostochiensis* in the roots of the resistant cultivars was within the known response to *H1* gene present in cv. 'Laura'. The resistance response is often characterized by a hypersensitive reaction and necrosis of cells surrounding the invading juveniles (Rice et al., 1985). The juveniles are unable to establish active syncytia and they either die, leave the root system or moult to males that survive malnutrition (Giebel, 1982; Forrest et al., 1986; Schouten, 1993; Moens et al., 2018). All the tested populations from Kenyan were avirulent on cv. 'Laura' and other clones containing the *H1* gene and, therefore, most likely belonged to pathotype Ro1/4 of *G. rostochiensis* (Kort et al., 1977; Mugniery et al., 1989). In addition, the Kenyan 'HAR1' and German 'Ecosse' population had similar hatching and penetration patterns in the roots of resistant cv. 'Laura' and susceptible cv. 'Désirée'. However, the Kenyan population required more degree-days (736 DD_6) compared to 'Ecosse' (645 DD_6) to complete its life cycle. Information on degree-days required by a population to complete the life cycle is important in PCN management (Ebrahimi et al., 2014). For example, the duration of life cycle is important when using trap crop in PCN management since farmers are able to destroy the trap crops before the life cycle is complete.

The study of six *G. rostochiensis* populations from Kenya revealed low genetic diversity (Chapter 3), that can be attributed to genetic drift and founder effects (Boucher et al., 2013). A high genetic diversity is found among PCN populations from South America (Grenier et al., 2010), the primary centre of PCN origin (Mai, 1977). It is postulated that only a small proportion of the South American diversity was introduced in to Europe (Plantard et al., 2008). As PCN spread from Europe to other parts of the world (Turner & Evasn, 1998), including Kenya, diversity may have further reduced. The overall low genetic differentiation among the populations as indicated by low F_{ST} values (Table 3.3) can be explained by significant gene flow between populations. Since PCN are not highly mobile, human-mediated dispersal plays a key role in genetic flow (Plantard & Porte, 2004). Farm practices of potato farmers in Kenya contribute to the gene flow. They share potato tubers and seeds (Muthoni et al., 2013), farm tools and machinery, and transportation of

uncleaned tubers, among others. However, the low genetic differentiation may also indicate a case of recent introduction.

It is unknown when *G. rostochiensis* was introduced into Kenya. It has been speculated that Kenyan PCN were of European ancestry for two reasons: First, Kenya has a long history of importing seed potatoes from Europe (Njoroge, 1982; Anonymous, 2016) thus increasing the possibility of passive spread through contaminated potato tubers. Second, this study showed that Kenyan populations belonged to the Ro1/4 pathotype group (Chapter 2), which is the predominant pathotype group in Europe. However, while the Kenyan populations were more similar to the world *G. rostochiensis* populations tested by Boucher et al. (2013) apart from two South American populations (Fig. 3.3a), they are distinct (Fig. 3.3b). Populations from Germany, where cyst nematodes were first reported (Moens et al., 2018), The Netherlands and UK which have a long history of supplying potatoes to Kenya (Njoroge, 1982; Anonymous, 2016), were not included in the Boucher et al. (2013) study and therefore not compared in this study. To ascertain the putative source of the Kenyan populations, a more detailed study should include more populations from Europe, particularly from countries that export seed potatoes and tubers to Kenya as well as populations from other countries in Africa. Nevertheless, this study generated important information regarding *G. rostochiensis* from Kenya, which is critical in designing a resistance-based management.

6.2 Resistance Management to avoid Selection for Increased Virulence

Sustained use of resistant cultivars in the management of PCN may lead to the selection and emergence of resistance breaking populations or selection in favour of another nematode species. For instance, the *H1* gene has been effective in the management of *G. rostochiensis* in the United Kingdom (UK) for over half a century (Bakker et al., 2006). However, continued cultivation of potato cultivars with the *H1* gene in fields infested with *G. rostochiensis* and *G. pallida* allowed for the selection of the latter, which is now the predominant species in the UK (Minnis et al., 2002). It takes only a few generations of reproduction on the same cultivar for *G. pallida* to adapt to QTLs and break the resistance (Eoche-Bosy et al., 2017). Considering the fact that *G. pallida* has been reported in Kenya already, there is reason to worry about such changes in response to the deployment of *H1* carrying potato cultivars against *G. rostochiensis.* A good example of the emergence of field selected *G. pallida* is the German resistance breaking *G. pallida* population,

'Oberlangen', which was first reported by Niere et al. in 2014 that was therefore used in this dissertation to better understand the changes that led to increased virulence.

Interestingly, the hatching behaviour and penetration of the 'Oberlangen' juveniles in the host roots could not account for the increased reproduction reported (Niere et al., 2014). However, the resistance mechanism in resistant cv. 'Seresta' restricted the development of the avirulent nematodes from the 'Chavornay' population while virulent individuals of the 'Oberlangen' population successfully reproduced on this cultivar (Chapter 4). Reproduction on a resistant cultivar occurs when the invading juveniles are able to induce active syncytia required for development into females (Castelli et al., 2005; Moens et al., 2018). Such nematodes use effectors to overcome the host's resistance, supress resistance response and induce the formation and maintenance of a syncytium (Jones & Mitchum, 2018). Virulent individuals in a population may arise following a single mutation at the *Avr* locus that enables them to avoid recognition by the host *R* genes encoded receptors (Leroy et al., 2014). However, field populations of PCN have a proportion of virulent alleles inherited from the original introduction (Bakker et al., 2006). Selection pressure imposed on these populations allows the proliferation of virulent alleles leading to emergence of resistance breaking populations. This may explain the emergence of 'Oberlangen' population. It was indicated in this study that newly reported virulent populations had bigger cysts compared to the non-selected populations. This confirms findings by Fournet et al. (2016) that increase in virulence is characterised by increase in cyst size. This information is critical in the monitoring of the change in virulence the populations of *G. rostochiensis* populations from Kenya.

Management of the Kenyan Population

Resistant potato cultivars are effective in their action, easy to use and pose no risk to the environment (Starr et al., 2013; Milczarek et al., 2014; Davies & Elling, 2015). This makes them preferred method of PCN management among the smaller holder potato farmers in Kenya. As demonstrated in this study, potato cultivars with major resistance genes, such as *H1* gene, offer absolute resistance to specific pathotypes of PCN (Bakker et al., 2003). In the current study, the *G. rostochiensis* from Kenya were found to be predominantly Ro1/4 pathotype group. Nearly all the cultivars tested in this study (see chapter 2) were resistant to these populations, but only three of these cultivars are available in Kenya. However, the Kenyan potato catalogue 2017 has listed potato cultivars with resistance to *G. rostochiensis*, but specific details on the target pathotype(s)

are missing for most of these cultivars (Anonymous 2017). Potato farmers from Kenya can utilize the cultivars with resistance against Ro1/4 pathotype while the efficacy of the remaining cultivars should be tested using the populations identified in this study and specific description of the cultivars provided so that growers can make informed choices.

Unfortunately, there are no cultivars in the Kenyan catalogue that are resistant to *G. pallida* (Anonymous, 2017). Therefore, adoption of resistant cultivars in the management of *G. rostochiensis* in fields with mixed population of *G. rostochiensis* and *G. pallida* may select and allow the increase of the latter. This has been reported in UK and The Netherlands (Minnis et al., 2002; Bakker et al., 2006). In addition, continued of resistant cultivars in the management of *G. rostochiensis* may lead to the selection and emergence of resistance breaking populations such as 'Oberlangen' (Niere et al., 2014).

To avoid that, the utilization of the available cultivars should be integrated with other pest management methods. This would improve the efficiency of PCN management and prolong the efficacy of the available resistant cultivars (Trudgill et al., 2014). An integrated PCN management approach is highly relevant in Kenya because potato production is very intensive and the climate conditions are favourable for PCN reproduction (Janssens et al., 2013). Among the control method recommended for Kenyan farmers include use of crop rotation, trap crop, reinforcement of quarantine measures in farm(s) where *G. pallida* has been reported and proper farm hygiene (López-Lima et al., 2013; Trudgill et al., 2014).

Proper crop rotation systems are able to reduce the PCN level in the soil and to maintain good soil health (Franco et al., 1999). In many countries, crops rotation cycles of between 4-7 years using host and non-host crops are used to lower the nematode density to non-damaging levels (Franco et al., 1999). Long rotation cycles may appear impractical for most of smallholder farmers in Kenya who produce mainly for subsistence use. Only approx. 5.5% of the farmers practice crop rotation with crops such as maize and beans (Muthoni et al., 2013). Well-designed rotation cycles using economically important food crops such as cereals (maize, wheat and barley), legumes (beans and pea) and vegetable (cabbage, carrots) etc. that grow well in the region (Muthoni et al., 2013) may gain acceptance among farmers. These non-host crops trigger spontaneous hatching followed by death of hatched juveniles and therefore reducing the inoculum in the soil (Rice, 1985; Devine et al., 1999).

Alternatively, potato growers can opt to use trap crops in their farms. These are host crops that are resistant to PCN but able to stimulate hatching of nematodes eggs. The hatched juveniles penetrate the host, but die within the roots since they are not able to establish a parasitic relationship with the host (Rodriguez-Kabana, 1992). Apart from lowering the nematode density in the soil, trap crops help in soil enrichment by the addition of biomass while some of them are food crops of economic value to the farmer. Several members of Solanum spp. have been used as trap crops, for example *Solanum sisymbriifolium* (Dias et al., 2012) and non-tuber bearing *S. nigrum* (Scholte, 2000a). Some species of *S. nigrum* are grown in Kenya and are used as indigenous vegetable (Chitambo et al., 2019). Some Solanum spp. were found to be resistant to Kenya PCN (Chitambo et al., 2019) and therefore they are suitable trap crops for use in the management. However, use of tuber bearing *Solanum* spp. as trap crop is not tenable since it can lead to an increase in nematode populations following untimely destruction of the crop (Scholte, 2000a). In addition, some tubers survive in the soil and emerge as volunteer crop in the subsequent season leading to further nematode reproduction (Scholte, 2000b).

The use of proper farm hygiene mitigates the spread of PCN within or between potato fields. Potato farmers in Kenya practice intensive production and have no access to certified potato seeds and therefore produce their own seeds or obtain them from the neighbours (Muthoni et al., 2013). Such human aided dispersal might allow the spread of *G. pallida* to areas where it has not been reported. The resistance screening programme described in this study (see chapter 4) would highly enhance breeding and thus production of adequate affordable seed potato for Kenyan farmers. A lot of sensitization to the farmers of the modes of nematode spread as well as available management options would highly enhance management of PCN in Kenya.

6.4 Conclusion

The present work provides detailed information about Kenyan populations of *Globodera rostochiensis*. This information opens opportunities for the management using resistant potato cultivars as well as research in other integrated management options using resistance as a key component. In addition, several *G. rostochiensis* populations from Kenya were isolated and multiplied in the glasshouse, these populations are suitable reference materials in future studies. Several resistant potato cultivars have been recommended for use by farmers in Kenya (Chapter 2). However, there is need for constant monitoring of *G. rostochiensis* populations in the field

especially dispersal, changes in population densities and changes in virulence as farmers deploy the recommended resistant cultivars. In addition, pre-emptive measures to avoid increase of *G. pallida* are crucial.

The study of *G. pallida* population from Kenya was not achievable in the scope of this study. However, one population was recently isolated and the multiplication and further testing is in progress. *G. pallida*, though reported in Kenya at low frequency, if not properly managed might present a bigger challenge in the future. Therefore, future work should focus on *G. pallida* management. Basic information on pathotype(s), biology and virulence of this population are required for proper management. In addition, future breeding should focus on producing cultivars resistant to this population since there are only few resistant cultivars on the market compared to *G. rostochiensis*. The resistance testing system developed in this study can be used to enhance the breeding process while the population isolated can serve as a reference. For the meantime, the available potato cultivars in Kenya should be assessed for possible resistance while the field where *G. pallida* was detected should be placed under strict quarantine monitoring and, if possible, there should be no further potato production until there is no more detection.

In conclusion, *G. rostochiensis* in Kenya can be effectively managed using resistant potato cultivars, but care should be taken not to select *G. pallida* in Kenya. Resistant crop cultivars should be used in an integrated system, which includes monitoring, crop rotation and trap cropping among others. There is a clear need to enhance breeding for resistance, especially against *G. pallida* and the emerging virulent types.

6.6 References

6.6 References

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Summary

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida* are important potato pests responsible for high yield losses globally. The ability of PCN to persist in the soil for a long time makes their management challenging and therefore these nematodes are under strict quarantine regulations in many countries. Nevertheless, both PCN species have recently been reported in potato cropping systems in Kenya. Resistant potato cultivars reduce nematode densities in the field below damage threshold levels and thus help mitigate yield losses. However, utilization of resistant potato cultivars is affected by lack of information about PCN populations present, lack of adequate resistant cultivars and fears of virulence selection. The main objective of this study was to investigate ways of enhancing the use of resistant potato cultivars in the management of PCN in Kenya.

One main task for the successful implementation of resistant potato cultivars is knowledge about the PCN population(s) present in the field. The Kenyan populations of *G. rostochiensis* were therefore studied in glasshouse and laboratory experiments, which confirmed that they lack obligatory diapause and are unable to reproduce on potato cultivars carrying the *H1* resistance gene resulting in the classification as Ro1/4 pathotype. Potato cultivars carrying this gene were found suitable for use in PCN management. However, care should be taken when using resistant cultivars in the management of *G. rostochiensis* in Kenya to avoid selection and proliferation of *G. pallida* which is present in the country, but still at low frequency.

Further studies showed that Kenyan populations of *G. rostochiensis* have low genetic variability. Heterozygote deficit was found in some of the populations that was attributed to bottleneck effects following recent introduction(s). The structure of these populations differed from all populations they were compared to and the putative source of Kenyan populations could not be ascertained in this study. Further investigations are needed including additional European populations and from around the world, to identify the routes of introduction of Kenyan PCN.

Studies of a selected virulent *G. pallida* population, ‘Oberlangen’, showed that the increase in virulence resulted from the ability of juveniles to moult and develop into females since the hatching and penetration of this population on resistant cultivars was similar to the avirulent reference population ‘Chavornay’. Differences in life history traits of ‘Oberlangen’ versus the reference population ‘Chavornay’ could not be attributed to increased virulence. However, comparison of

cyst diameters of virulent and avirulent populations showed a positive correlation with virulence. Cultivars tested for potential resistance to 'Oberlangen' were susceptible, but suppressed the reproduction of 'Chavornay'. Therefore, there is a need for enhanced breeding for resistance to the new virulence type 'Oberlangen' and other virulent populations reported. 'Oberlangen' can serve as a future reference population for testing breeding materials for resistance to the new *G. pallida* virulence type.

To enhance the breeding process, tissue culture (TC) derived potato plants were evaluated in phenotyping experiments. The TC derived plants supported the reproduction of PCN and they were therefore suitable and highly effective for resistance screening. Resistance assessment can be done in different pot volumes, provided the average inoculum density of 5 eggs and J2s ml^{-1} soil is applied within seven days of planting. Furthermore, the results obtained with TC plants were comparable with those obtained using the EPPO standard protocol. Visual assessment of white females on the pot surface could be a quick way of identifying susceptible genotypes that can then be discarded. Tissue culture plants can enhance the screening process and significantly lower the associated cost making them for a valuable tool for enhancing the breeding process in Kenya.

In conclusion, this study has generated important information about the Kenyan populations of *G. rostochiensis* in support of a sustainable management. If used properly, resistant potato cultivars can manage these populations and selection of virulent populations can be avoided. Furthermore, *G. pallida* must be contained to reduce the risk of spreading throughout Kenya. To achieve this, monitoring, quarantine measures, crop rotation and trap cropping amongst others should be enforced. Lastly, the screening test system developed in this study will goes a long way in enhancing breeding for PCN resistance.

Zusammenfassung

Die Kartoffelzystennematoden *Globodera rostochiensis* und *G. pallida* sind bedeutende Schädlinge für Kartoffeln und weltweit verantwortlich für hohe Ertragsverluste. Die Fähigkeit lange im Boden überdauern zu können macht ihr Management schwierig und führt daher zu strikten Regulierungen als Quarantäneorganismen weltweit. Beide Arten wurden kürzlich in den Kartoffelanbaugebieten in Kenia nachgewiesen. Da die Kartoffelproduktion in Kenia primär auf Kleinstfarmen stattfindet ist die Durchsetzung von Quarantänemaßnahmen kaum möglich, wodurch für die Nematoden optimale Bedingungen für eine Ausbreitung herrschen. Kartoffelsorten mit einer Resistenz gegen Kartoffelzystennematoden reduzieren nachhaltig die Nematodendichten auf ein Niveau unterhalb der Schadensschwelle und verhindern so Ertragsverluste. Der Erfolg des Einsatzes solcher resistenten Sorten wird jedoch von der vorhandenen Nematodenpopulation, der Verfügbarkeit geeigneter Sorten und der Gefahr der Selektion auf neuer Virulenzen beeinflusst.

Ziel dieser Arbeit war es daher die Eignung von resistenten Kartoffelsorten zum Management von Kartoffelzystennematoden in Kenia eingehender zu untersuchen. Ein wichtiger Faktor zur erfolgreichen Einführung resistenter Kartoffelsorten sind die biologischen Eigenschaften der in einer Fläche vorhandenen Nematodenpopulation(en). Die kenianischen *G. rostochiensis* Populationen wurden daher in Labor- und Gewächshausversuchen eingehender charakterisiert. Es zeigte sich, dass sie sich ohne die sonst obligatorische Diapause vermehren können und Sorten welche das H1 Resistenzgen tragen, nicht anfällig sind. Somit konnten die getesteten Populationen der Virulenzgruppe Ro 1/4 zugeordnet werden.

Verschiedene Sorten mit H1 Resistenzgen waren zum Management der kenianischen Kartoffelzystennematoden geeignet. Nichtsdestotrotz sollte beim Einsatz von resistenten Sorten zum Management von *G. rostochiensis* darauf geachtet werden, dass die bisher nur vereinzelt vorkommende Art *G. pallida* sich nicht weiter ausbreiten kann und in den Anbaugebieten etabliert.

Weitere Studien zeigten, das kenianische *G. rostochiensis* Populationen nur eine geringe genetische Variabilität aufwiesen. In einigen Populationen wurde ein heterozygotes Defizit nachgewiesen, was wahrscheinlich durch den Flaschenhalseffekt nach erst kürzlich erfolgten Einschleppungen verursacht wurde. Die Struktur dieser Populationen unterschied sich von

Europäischen Referenzpopulationen wodurch mögliche Einschleppungswege nicht nachvollzogen werden konnten. Weitere Untersuchungen, unter Einbindung weiterer Populationen aus Europa und anderen geographischen Regionen, sind nötig, um mögliche Einschleppungswege nach Kenia zu identifizieren.

Untersuchungen zu einer virulenten *G. pallida* Population ‚Oberlangen' zeigten, dass eine erhöhte Virulenz die Entwicklung der Juvenilen zu Weibchen ermöglichte, in Bezug auf den Schlupf und die Eindringung in resistente Kartoffelsorten aber kein Unterschied zur avirulenten Referenzpopulation ‚Chavornay' vorhanden war. Unterschiede bezüglich verschiedenen ‚Life-History-Traits' der Population Oberlangen im Vergleich zur Referenzpopulation ‚Chavornay' waren nicht auf eine veränderte Virulenz zurückzuführen. Einzig der Durchmesser der Zysten von virulenten gegenüber avirulenten Populationen zeigte einen positiven Zusammenhang. Kartoffelsorten, die eine Resistenz gegenüber der Referenzpopulation ‚Chavornay' besaßen, waren alle anfällig gegenüber der Population ‚Oberlangen'. Somit besteht ein großer Bedarf an Resistenzquellen zur Züchtung neuer, resistenter Sorten, die in entsprechenden Bekämpfungsprogrammen zum Management von *G. pallida* Populationen mit veränderter Virulenz eingesetzt werden können. Die eingehend untersuchte Population ‚Oberlangen' kann dabei als neue Referenz für diesen neuen Virulenztyp dienen.

Gewebekulturpflanzen können den Züchtungsprozess unterstützen und wurden daher in Phänotypisierungsexperimenten untersucht. Es zeigte sich, dass Kartoffelzystennematoden sehr gute Vermehrungsraten aufwiesen, was ein effektives Screening neuer Resistenzquellen ermöglicht.

Die Untersuchung einer Resistenz kann in Versuchsgefäßen mit unterschiedlichen Volumen bei einer Inokulumdichte von 5 Eiern und Juvenilen/ml^{-1} Boden und Inokulation sieben Tage nach der Pflanzung durchgeführt werden. Des Weiteren wurde mit Gewebekulturpflanzen identische Ergebnisse bezüglich der Resistenzbewertung nach EPPO Standard Protokoll erzielt werden. Zusätzlich kann die visuelle Bonitur von neugebildeten weißen Weibchen an der Außenseite der Versuchsgefäße als Schnelltest zur Identifikation und Selektion von anfälligen Genotypen genutzt werden. Der Einsatz von Gewebekulturpflanzen fördert den Phänotypisierungsprozess und verringert gleichzeitig die Kosten und stellt somit ein wichtiges Instrument für die

Züchtungsprogramme in Kenia dar. Diese Arbeit liefert wichtige Informationen bezüglich der kenianischen *G. rostochiensis* Populationen als Grundlage für ein nachhaltiges Management unter Vermeidung der Selektion von neue virulenten Populationen. Richtig eingesetzt können resistente Kartoffelsorten zur Regulierung von *G. rostochiensis* verwendet und die Selektion virulenter Populationen vermieden werden. Die Etablierung von *G. pallida* muss unter Einhaltung von Quarantänebedingungen, Monitoring, Fruchtfolgemaßnahmen, Fangpflanzen und andere Methoden verhindert werden. Das entwickelte Testsystem wird zukünftig den Weg der Züchtung neuer Sorten in Kenia mit Resistenz gegen Kartoffelzystennematoden nachhaltig unterstützen.

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Braunschweig 24th October, 2019

James Maina Mwangi