GENETICS OF VIRULENCE IN POTATO CYST NEMATODES

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op woensdag 7 februari 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Offset printed by Ponsen & Looijen, Wageningen

Cover design: Frederik J.J. von Planta
Design Studio - Gecombineerde Diensten Binnenhaven
Agricultural University Wageningen

This thesis has been accomplished at:
Department of Nematology
Agricultural University
Binnenhaven 10
6709 PD Wageningen
The Netherlands

STELLINGEN

1. Het ontbreken van duidelijke Z-lijnen in de lichaamswand-musculatuur van Caenorhabditis elegans verklaart waarom in deze nematode de "gap-filament" eiwitten nebuline en titine niet of nauwelijks waarneembaar zijn.
   Francis, G.R. & Waterston, R.H. (1985). J. Cell Biol., 101: 1532-1549.
   Hu, D.H., Kimura, S.& Maruyama, K. (1986). J. Biochem., 99: 1485-1492.
   Wang, K. & Wright, J. (1988). J. Cell Biol., 107: 2199-2212.

2. Hoewel de positieve reactie van enkele nucleïnezuurkleuringen met secretie-blaasjes in de speekselklier van Meloidogyne incognita zou kunnen wijzen op een uniek en revolutionair celbiologisch proces, moet de verklaring voor dit verschijnsel eerder gezocht worden in de aspecificiteit van de gebruikte cytochemische technieken.
   Sundermann, C.A. & Hussey, R.S. (1988). J. Nematol., 20: 141-149.

3. Indien bloei-inductie bij de aardappel ook plaatsvindt bij aantastingen door aardappelcysteaaltjes, is hiermee een verklaring gegeven voor het grote aantal zelf-incompatibele diploïde aardappelsoorten met aardappel-cysteaaltjes resistentie.
   Hawkes, J.G. (1978). In: Harris, P.M. (Ed.), Potato Crop, 15-69.
   Simmonds, N.W. (1976). In: Simmonds, N.W. (Ed.), Evolution of Crop Plants, 279-283.
   Sidhu, S & Webster, J.H. (1981). In: Zuckerman, B.M. & Rohde, R.A. (Eds.), Plant Parasitic Nematodes, Vol. III, 61-87.

4. "Photobiochemistry in the dark" is een belangrijk proces voor de in vivo nematicide activiteit van thiofenen en thiarubrines en niet voor de nematicide polyacetylenen.
   Cilento, G. (1980). Photochem. Photobiol. Rev., 5: 199.
   Cilento, G. (1984). Pure Appl. Chem., 56: 1179.
   Towers, G.H.N. & Champagne, D.E. (1988). In: Lam, J., Breteler, H., Arnason, T. & Hansen, L. (Eds.), Bioactive Molecules, Vol. VII, 139-151.

5. Hoge concentraties milieuvreemde stoffen in topcarnivoren kunnen met evenveel recht verklaard worden door allometrische modellen zonder voedselpyramide structuur.
   Griesbach, S., Peters, R.H. & Youakim, S. (1982). Can. J. Fish. Aquat. Sci., 39: 727-735.

6. Het manipuleren met DNA verstoort de altruïstische spelletjes van egoïstische genen.
   Barash, P.D. (1987). In: The Hare and the Turtle.

7. Het gebruik van "Reduced Instruction Set" versnelt niet alleen de werking van microprocessoren, maar verklaart ook de hoge reproductie-snelheid van micro-organismen.

Stellingen behorende bij het proefschrift, getiteld:
"Genetics of virulence in potato cyst nematodes" door Richard Janssen.

Wageningen, 7 februari 1990.

Voor

A.J.C. Janssen-Suyderhoud
Marja Morskieft
Kiki Maria †
Bo Ramon

VOORWOORD

Het verschijnen van dit proefschrift biedt een goede gelegenheid om mijn begeleider, dr. Fred J. Gommers, speciaal te bedanken voor de wijze waarop hij mij in alle vrijheid aan dit onderzoek heeft laten werken en mijn collega, dr. Jaap Bakker, voor de wetenschappelijke en tegelijkertijd informele samenwerking die uiteindelijk heeft geresulteerd in de totstandkoming van dit proefschrift.

Ik dank prof. A.F. van der Wal voor zijn bereidwilligheid als promotor te fungeren.

Prof. E. Jacobsen, dr. D. Mugniery (Rennes, Frankrijk), dr. P. Stam en prof J.C. Zadoks ben ik erkentelijk voor hun kritische opmerkingen en ik dank dr. R.N. Perry (Harpenden, Engeland) en dr. W.M. Wouts (Auckland, Nieuw-Zeeland) voor het corrigeren van een aantal manuscripten.

Medewerkers van de Vakgroep Nematologie en Gecombineerde Diensten, collega's van het Instituut voor Planteziektenkundig Onderzoek, de Plantenziektenkundige Dienst, de Stichting voor Plantenveredeling en het Hilbrands Laboratorium, en studenten dank ik in het bijzonder voor al hun bewezen diensten.

Het aardappelbedrijfsleven heeft, mede door de inspanningen van dr. D.E. van der Zaag, voorzitter van de Nederlandse Aardappelassociatie (NAA), dit onderzoek mogelijk gemaakt door het vooronderzoek (1981-1984) financieel te ondersteunen via het 'Reuzecelproject', en het vervolgonderzoek (1984-1989) via de Werkgroep Aardappelcysteaaltjesonderzoek (WACO).

Richard Janssen

Wageningen
Oktober, 1989

CONTENTS

INTRODUCTION

In 1536 when the conquest of Peru was being accomplished an expedition set out from Santa Marta on the Caribbean shore of what is now called Colombia. The leader was Jiménez de Quesada and the expedition worked its way south out of the Magdalena valley. They saw for the first time potatoes in the Valle de la Grita of the northern Andes, in the modern province of Velez (Hawkes, 1967).

The nearest estimate of the date of introduction in Europe is 1570 in Seville, Spain. From Spain the potato spread into continental Europe. At a later date 1596 potatoes were introduced in Ireland and from there in Scotland and England. The potato was probably taken to North America in 1719 by Scottish or Irish immigrants who established a colony at Londonderry, New Hampshire. Social changes helped to overcome objections to the crop and fostered its acceptance. By the nineteenth century its use was widespread (Dodds, 1966).

Potato cyst nematodes have co-evolved with their solanaceous hosts in the Andean region of South America and were introduced in Europe with potatoes imported for breeding purposes from about 1850 onwards (Jones & Jones, 1974). Today potato cyst nematodes are present in nearly all temperate zones where potatoes are cultivated and they embody a considerably loss to the farmer. At a world scale these losses are estimated to constitute about 10% of the annual yield (Brodie, 1984). In 1923, Wollenweber classified the potato strain of the sugar beet cyst nematode Heterodera schachtii (Schmidt) as the potato cyst nematode Heterodera rostochiensis. The first sign that the potato cyst nematode might not be a single species was the discovery of populations able to overcome certain genes for resistance (Jones et al., 1970). It became apparent that not all pathotypes interbreed freely and that in Europe two sibling species of potato cyst nematodes are present: Globodera rostochiensis (Skarbilovich [Woll.]) and G. pallida (Stone) (Stone, 1972). The late discovery of the two species resulted from their striking morphological similarity, but on the

basis of variations in proteins, they are estimated to have diverged millions of years ago (Bakker, 1987).

Control of potato cyst nematodes is based on combinations of (1) crop rotation, (2) soil disinfestation with various nematicides and, (3) the use of resistant potato varieties. In the near future the application of nematicides may be restricted and the control of potato cyst nematodes will rely more on crop rotation and the use of resistant potato varieties. Both Globodera species have their own virulence spectra with respect to genes for resistance. A simple and quick screening test to characterize and quantify field populations according to species would create the possibility to develop potato varieties with a limited but defined spectrum of resistance. This consideration led to the development of monoclonal antibodies able to discriminate potato cyst nematodes at the species level (Schots, 1988).

Table 1 International scheme for recognition of pathotypes of Globodera rostochiensis and G. pallida (Kort et al., 1977)

Clone	Pathotypes							
	Ro_1	Ro_2	Ro_3	Ro_4	Ro_5	Pa_1	Pa_2	Pa_3
S. tuberosum ssp. tuberosum	+	+	+	+	+	+	+	+
S. tuberosum ssp. andigena CPC 1673 (gene H_1)	-	+	+	-	+	+	+	+
S. kurtzianum hybr. 60.21.19 (gene A,B)	-	-	+	+	+	+	+	+
S. vernei hybr. 58.1642/4	-	-	-	+	+	+	+	+
S. vernei hybr. 62.33.3	-	-	-	-	±	-	-	+
S. vernei hybr. 65.346./19	-	-	-	-	-	+	+	+
S. multidissectum hybr. P55/7 (gene H_2)	+	+	+	+	+	-	+	+
S. vernei hybr. 69.1377/94	-	-	-	-	-	-	-	-

+ = $P_f/P_i > 1$; - = $P_f/P_i \leq 1$

Currently eight pathotypes are recognized in Europe (Table 1), five within G. rostochiensis (Ro_1 - Ro_5) and three within G. pallida (Pa_1 - Pa_3) (Kort et al., 1977). Canto Saenz & de Scurrah (1977) set up a similar Andean scheme with other denominations and detected several pathotypes not present in Europe. The European pathotypes are defined by their multiplication rates on six differential clones: Solanum tuberosum ssp. andigena CPC 1673 (Ellenby, 1952), S. kurtzianum hybrid 60.21.19 (Huijsman, 1960), the S. vernei accessions GLKS 58.1642/4, 62.33.3 and MPI 65.346/19 (e.g. Ross, 1969; Huijsman & Lamberts, 1972; Kort et al., 1972) and S. multidissectum hybrid P55/7 (Dunnett, 1961). Resistance against potato cyst nematodes is also found in other solanaceous species (e.g. Sidhu & Webster, 1981; Ross, 1986). Usually P_f/P_i values are determined in pot experiments by inoculating approximately 25 cysts (P_i) and assessing the number of newly developed cysts (P_f). These types of experiments can also be carried out in closed containers (Foot, 1977).

The definition of pathotype commonly used in phytopathology is not valid for potato cyst nematodes. Pathotypes are classified as virulent or avirulent for a differential if P_f/P_i-values are respectively >1 or ≤ 1. This implies that pathotypes are not necessarily fixed for alleles for virulence or avirulence. As a consequence, populations classified as identical pathotypes may vary in the number of virulent individuals for a given differential (Bakker, 1987). In this thesis the term virulent individual is reserved for the ability to overcome resistance and to develop into a female.

Multiplication rates as such are not an appropriate measure for the level of virulence in a population. P_f/P_i values are density dependent and influenced by differences in hatching (Forrest & Phillips, 1984; Rawsthorne & Brodie, 1986). Furthermore P_f/P_i values are extremely sensitive to environmental factors and often result in large variation between and within experiments. A more accurate method to estimate the frequencies of virulent genotypes is to express the P_f/P_i on a differential as a percentage of the P_f/P_i on a general susceptible host. However, near isogenic lines with and without resistance do not exist. This implies that rates of multiplication are also influenced by genes other than those for resistance.

Problems associated with pot experiments and the lack of near isogenic lines can be minimized by rearing cysts on roots of sprouts grown in Petri dishes (Mugniery & Person, 1976). This method minimizes variations due to e.g. differences between cultivars in rates of root growth, hatching or inoculum densities per root tip, and allows therefore more accurate estimates of the number of virulent phenotypes. In addition this method can be used for controlled matings between females and single males which allows a precise genetic analysis.

Howard (1959) and Jones & Parrott (1965) suggested that similar to the system of Linum usitatissimum-Melampsora lini, the interaction between G. rostochiensis and the H_1 gene for resistance of Solanum tuberosum ssp. andigena CPC 1673 is also controlled by a gene-for-gene mechanism with virulence (a) being inherited at a single locus and recessive to avirulence (A). It was also assumed that this interaction is confined to larvae developing into females and that males developing on a resistant clone can have any genotype (AA, Aa or aa). Studies to support this gene-for-gene hypothesis (Parrott & Berry, 1974; Parrott, 1981) lacked the experimental and numerical base necessary for a formal proof.

Several sources of resistance against potato cyst nematodes were investigated. Resistance from S. tuberosum ssp. andigena CPC 1673 (gene H_1) (Ellenby, 1952), S. kurtzianum (gene A,B) (Huijsman, 1960) and S. multidissectum (gene H_2) (Dunnett, 1961) is based on major genes whereas the resistance in the S. vernei accessions (e.g. Ross, 1969; Huijsman & Lamberts, 1972; Kort et al., 1972) has a polygenic nature. The most important sources of resistance used in Western Europe are those from S. tuberosum ssp. andigena and S. vernei. Because of the monogenic nature of the H_1 resistance (Huijsman, 1955) it was decided to study the virulence in G. rostochiensis for this gene. The main objectives of this study were to obtain lines of G. rostochiensis with defined levels of virulence and to investigate the inheritance of virulence. This kind of information will contribute to a better understanding of virulence in field populations and will also create possibilities to study the virulence mechanisms at the molecular level.

Therefore (i) an accurate method was developed to estimate as precisely as possible intra-specific variations in virulence in populations of G. rostochiensis and G. pallida (chapter II), (ii) a method was found to circumvent the diapause of potato cyst nematodes (chapter I), (iii) avirulent and virulent lines were selected after controlled single matings in Petri dishes (chapter III), (iv) the inheritance of virulence was determined for the resistance gene H_1 of S. tuberosum ssp. andigena CPC 1673 (chapter IV), and (v) the effect of heterozygosity for virulence on the development of potato cyst nematodes was analysed (chapter V).

References

Bakker, J. (1987). Protein Variation in Cyst Nematodes. Ph.D. Thesis, Univ. of Agriculture, Wageningen, The Netherlands, 159 pp.

Bakker, J. & Bouwman-Smits, L. (1987). Contrasting rates of protein and morphological evolution in cyst nematode species. Phytopathology, 78: 900-904.

Brodie, B.B. (1984). Nematode parasites of potato. In: Nickle, W.R. (Ed.). Plant and Insects Nematodes, Marcel Dekker Inc., New York: 167-212.

Canto Saenz, M. & de Scurrah, M.M. (1977). Races of potato cyst nematode in the Andean region and a new system of classification. Nematologica, 23: 340-349.

Dodds, K.S. (1966). The evolution of the cultivated potato. Endeavour, 25: 83-88.

Dunnett, J.M. (1961). Inheritance of resistance to potato-root eelworm in breeding lines stemming from Solanum multidissectum Hawkes. Rep. Scottish Breed. Stn.,: 39-46.

Ellenby, C. (1952). Resistance to the potato root eelworm, Heterodera rostochiensis Wollenweber. Nature, 170: 1016.

Foot, M.A. (1977). Laboratory rearing of potato cyst nematode; a method suitable for pathotyping and biological studies. N.Z. J. Zool., 4: 183-186.

Forrest, J.M.S. & Phillips, M.S. (1984). The effect of Solanum tuberosum x S. vernei hybrids on hatching of the potato cyst nematode Globodera pallida. Ann. appl. Biol., 104: 521-526.

Hawkes, J.G. (1966). The history of the potato, part I. J. Roy. Hort. Soc., 92: 207-224.

Howard, H.W. (1959). Biotypes of potato root eelworm in Great Britain. Tagber. Dt. Akad. Landw-Wiss. Berl., 20: 95-106.

Huijsman, C.A. (1955). Breeding for resistance to the potato root eelworm II. Euphytica, 4: 133-140.

Huijsman, C.A. (1960). Some data on the resistance against the potato root-eelworm (Heterodera rostochiensis W.) in Solanum kurtzianum. Euphytica, 9: 185-190.

Huijsman, C.A. & Lamberts, H. (1972). Breeding for resistance to the potato cyst-nematode in the Netherlands. In: French, E.R. (Ed.). Prospects for the Potato in the Developing World, Int. Potato Center, Lima, Peru: 161-171.

Jones, F.G.W., Carpenter, J.M., Parrott, D.M., Stone, A.R. & Trudgill, D.L. (1970). Potato cyst nematode: one species or two? Nature, 227: 83-84.

Jones, F.G.W. & Parrott, D.M. (1965). The genetic relationships of pathotypes of Heterodera rostochiensis Woll. which reproduce on hybrid potatoes with genes for resistance. Ann. appl. Biol., 56: 27-36.

Jones, F.G.W. & Jones, M.G. (1974). Pests of Field Crops. 2nd edition, Edward Arnold, London, 448 pp.

Kort, J., Jaspers, C.P. & Dijkstra, D.L. (1972). Testing for resistance to pathotype C of Heterodera rostochiensis and the practical application of Solanum vernei hybrids in the Netherlands. Ann. app. Biol., 71: 289-294.

Kort, J., Ross, H., Rumpenhorst, H.J. & Stone, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst-nematodes Globodera rostochiensis and G. pallida. Nematologica, 23: 333-339.

Mugniery, D. & Person, F. (1976). Méthode d'elevage de quelques nématodes à kystes du genres Heterodera. Sci. Agron., Rennes, 217-220.

Parrott, D.M. (1981). Evidence for gene-for-gene relationships between resistance gene H_1 from Solanum tuberosum ssp. andigena and a gene in Globodera rostochiensis, and between H_2 from S. multidissectum and a gene in G. pallida. Nematologica, 27: 372-384.

Parrott, D.M. & Berry, M.M. (1974). Inheritance of ability to overcome resistance. Rothamsted Exp. Stn. Rep. 1973, Part 1: 152-153.

Rawsthorne, D. & Brodie, B.B. (1986). Relationship between root growth of potato, root diffusate production, and hatching of Globodera rostochiensis. J. Nematol., 18: 379-384.

Ross, H. (1969). Zuchtung von Kartoffelsorten mit Resistenz gegen Heterodera-rostochiensis Woll. Mitt. Biol. Bundesanst. Land. Forstwirtsch. Berlin-Dahlem, 136: 59-64.

Ross, H. (1986). Potato Breeding - Problems and Perspectives. In: Horn, W. & Röbbelen, G. (Eds.). Advances in Plant Breeding, J. Plant Breed., suppl. 13, Paul Parey, Berlin, 132 pp.

Schots, A. (1988). A Serological Approach to the Identification of Potato Cyst Nematodes. Ph. D. Thesis. Univ. of Agriculture, Wageningen, The Netherlands, 118 pp.

Sidhu, G.S. & Webster, J.M. (1981). Genetics of plant-nematode interactions. In: Zuckerman, B.M. & Rohde, R.A. (Eds.). Plant Parasitic Nematodes, Vol. III, Academic Press, Inc., New York: 61-87.

Stone, A.R. (1972). Heterodera pallida n. sp. (Nematoda: Heteroderidae), a second species of potato cyst nematode. Nematologica, 18: 591-606.

CHAPTER I

CIRCUMVENTING THE DIAPAUSE OF POTATO CYST NEMATODES

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Abstract

The diapause of potato cyst nematodes was bypassed by avoiding desiccation of the cysts. Larvae were artificially hatched by cutting the cysts in halves and subsequent incubation in potato root diffusate. Approximately 40% of the cyst content hatched. These treatments had no influence on viability and fecundity as ascertained by rearing nematodes in pots and on roots of sprouts grown on water agar in Petri dishes. With the artificial hatching procedure it is possible to produce five to six generations a year in Petri dishes and three to five generations in pots.

Introduction

In the international pathotype scheme of Kort et al. (1977) potato cyst nematode populations are designated virulent or avirulent for a given differential if the multiplication factor (P_f/P_i) is > 1 or ≤ 1, respectively, implicating that populations of pathotypes are defined as a response to a certain resistance source. In compatible combinations the multiplication factors may range from 1 to approximately 40 indicating that the numbers of virulent individuals

vary considerably among populations classified as identical pathotypes. Generally those variations for virulence are not taken into account in studying the biochemical and histopathological aspects of the host-parasite interactions. Moreover, current knowledge on the genetics of the host-parasite interaction is inadequate in obtaining conclusive results.

Genetic research is hampered by the diapause. Preventing the potato cyst nematodes from going into diapause will accelerate genetic studies on the Globodera rostochiensis (Woll.) Behrens and G. pallida (Stone) Behrens pathotypes. Although a substantial body of literature is available on the hatching process (e.g. Clark and Perry, 1977) no detailed information is published on bypassing the diapause. There are reports which point at this problem. Cunningham (1960), Ellenby and Smith (1967), Guile (1967) and Rode (1971) showed that if there is a diapause it varies considerably in length. Shepherd and Cox (1967) found that fresh brown cysts, if crushed, gave a 39% hatch of the total cyst content. Promising results were also obtained by McKenna and Winslow (1972) who managed to produce six generations a year by subsequent inoculation of pots with soil containing cysts of the former generation.

Preliminary experiments involving temperature shocks were not satisfactory. Storing moist and air-dried cysts during various alternating time intervals at -80°, -20°, +4° and 21°C did not result in any significant acceleration of the hatching process.

The research described in the present paper investigates the possibilities to accelerate genetic research by shortening the time between subsequent generations.

Materials and methods

The G. rostochiensis populations Ro_1-Mierenbos, Ro_3-C_{133}, Ro_4-F_{515} and G. pallida populations Pa_2-Dutch D and Pa_3-E_{1202} were obtained from the Plant Protection Service (Wageningen, the Netherlands). Ro_5-Harmerz was from Dr H.J. Rumpenhorst (Münster, Federal Republic of Germany) and Pa_2-HPL_1 from Ir A. Mulder (Hilbrands Laboratorium, Assen, the Netherlands). The pathotype classification of G.

rostochiensis (Ro_1-Ro_5) and G. pallida (Pa_1-Pa_3) are according to the scheme of Kort et al. (1977) and the codes refer to the registration in the original collections. The populations were maintained on Solanum tuberosum ssp. tuberosum L. 'Eigenheimer' susceptible to all pathotypes.

Females were reared on roots of sprouts grown on water agar in Petri dishes (Mugniery and Person, 1976; Mugniery, 1982). Two second-stage larvae were inoculated per root tip and incubated at 21°C. Four to six weeks after inoculations each female was mated with a single male. The males from the seven populations were reared in pots on 'Eigenheimer' grown in sand in a climate chamber at 21°C. A slow release N-P-K fertilizer (Osmocote ^{R}) was added. One month after inoculation the males were recovered from the soil with an Oostenbrink elutriator (Oostenbrink, 1960).

In pot experiments with three G. rostochiensis populations young cysts were reared on 'Eigenheimer' as described above for males. Six, 8 and 12 weeks after inoculation the young wet cysts were picked from the rootballs or collected from the soil with a Kort elutriator (Kort, 1960).

Root diffusate was obtained by placing a tuber of S. tuberosum ssp. tuberosum L. 'Bintje' in the dark, above a beaker filled with tap water. Approximately one month later, when the roots were developed, the solution was filtered, stored at 4°C and used as hatching agent.

Hatching tests were performed in micro-titer plates (Linbro ^{R}) with one cyst per hole (25 mm^3) at 21°C. The hatching agent was refreshed weekly.

Results

Artificial hatching from newly formed fresh cysts reared in Petri dishes

The percentage of inoculated second-stage larvae of the G. rostochiensis and G. pallida populations that developed into females ranged from 52-84%. The number of fertilized females obtained after single matings varied from 33-60% of the larvae inoculated. The

average content was 193 eggs per cyst. The number of successful matings expressed as a percentage of the total number of females obtained was 59% on the average for the G. rostochiensis populations and 68% for the G. pallida populations (Table 1).

Table 1. Numbers of nematodes and hatched second-stage larvae (F_1 generation) from newly formed cysts obtained 10-12 weeks after controlled inoculations and singular matings (P generation) on roots of potato sprouts grown on water-agar in Petri dishes.

Pathotype population	P					F_1			
	number of inoculated larvae[1]	females developed		fertilized females		number of eggs/cyst	artificial hatch[3] 2 days	2 weeks	1 month	
		No.	(%)[2]	No.	(%)[2]		No.	No.	No.	(%)[4]
Ro_1-Mierenbos	104	73	(70)	47	(45)	156	18	52	75	(48)
Ro_3-C_{133}	102	86	(84)	43	(42)	120	12	52	58	(48)
Ro_4-F_{515}	90	63	(70)	36	(40)	205	39	66	69	(33)
Ro_5-Harmerz	90	71	(79)	47	(52)	151	27	83	97	(64)
Pa_2-Dutch D	120	62	(52)	46	(38)	180	21	56	72	(40)
Pa_2-HPL_1	102	80	(78)	61	(60)	244	12	41	110	(45)
Pa_3-E_{1202}	100	60	(60)	33	(33)	297	16	119	127	(42)
LSD (P<0.05)									6.10	

[1] Obtained from air-dried 1 year old cysts.

[2] Percentage of inoculated larvae.

[3] Cumulative number of hatched larvae; mean of 30 cysts.

[4] Percentage of average number of eggs per cyst.

From the cysts thus obtained 30 per population were used in an artificial hatching test immediately and the remainders were stored air-dry and tested for hatchability after one year. Artificial hatching was performed by cutting cysts in halves with a scalpel and subsequent incubation in root diffusate. Hatched larvae were counted after two days, two weeks and one month (Table 1).

Table 2. Numbers of nematodes and hatched second-stage larvae (F_2 generation) from newly formed cysts obtained 10-12 weeks after controlled inoculations and singular matings (F_1 generation) on roots of potato sprouts grown on water-agar in Petri dishes.

Pathotype population	F_1			F_2	
	number of inoculated larvae	females developed No.(=%)[1]	fertilized females No.(=%)[1]	number of eggs/cyst	artificial hatch[2] No. (%)[3]
Ro_1-Mierenbos	100	66	40	127	50 (39)
Ro_3-C_{133}	100	67	56	169	96 (57)
Ro_4-F_{515}	100	57	—[4]	---	-- --
Ro_5-Harmerz	100	69	45	153	58 (38)
Pa_2-Dutch D	100	57	—[4]	---	-- --
Pa_2-HPL_1	100	69	46	201	98 (49)
Pa_3-E_{1202}	100	51	—[4]	---	-- --
LSD (P<0.05)					7.02

[1] Percentage of inoculated larvae.

[2] Mean of 30 cysts; hatch determined after 1 month.

[3] Percentage of average number of eggs per cyst.

[4] No males for fertilization.

In one month about 46% of the total cyst content hatched (Table 1). Cysts from the same batches stored air-dry for one year - a standard method - gave an average hatch of 71% when the whole cysts were incubated in root diffusate.

As shown in Table 2 the viability and fecundity of females from the artificially hatched larvae was not significantly affected. The development, the fertilization as well as the egg content of the females were for all populations in the same range as those of the starting generation.

Using the method outlined here we were able to produce four to five generations a year. In two weeks sufficient larvae were obtained by artificial hatching to start a new generation. It took 8 to 10 weeks to obtain fertile females and two additional weeks for the eggs to develop from which the second-stage larvae could be artificially hatched again.

Artificial hatching from newly formed fresh cysts reared in pots

Subjected to artificial hatching, on an average 68 active second-stage larvae were obtained per brown cyst of G. rostochiensis recovered from the roots 12 weeks after inoculation. This figure corresponds with 40% of the total egg content. For comparison, a number of the cysts was stored air-dry for a year and then placed in hatching agent. The number of active larvae thus obtained averaged 75% of the cyst content (Table 3).

Evidently, cutting of cysts in halves as well as moisture conditions favour the hatchability. When 12-week-old fresh brown cysts were placed in hatching agent without cutting them the hatchability decreased to about 3% of the cyst content (Table 3). Also when 12-week-old fresh brown cysts were stored air-dry during two weeks, cut in halves and placed in hatching agent the percentage of active larvae obtained dropped to 12% as compared to 40 % for artificial hatching.

To investigate possibilities to obtain several generations a year in pot experiments, population Ro_3-C_{133} was studied in detail. Crushed young cysts were inoculated two weeks after planting potatoes in pots. Ten to 14 weeks later new cysts were harvested and inoculated again as

described above. Using this method with an initial density of 1 cyst per 10 g soil the multiplication factor of the four generations was on an average 16.4 per generation.

Table 3. Effect of cyst age on artificial and normal hatching of active second-stage larvae from newly formed cysts and 1 year old air-dry stored cysts reared on potato roots grown in pots. Numbers of larvae are averages of 60 cysts after one month of incubation.

Pathotype population	Age (weeks after inoculation)	Cyst[1] colour	Number of eggs/ cyst	Hatching from new cysts		Normal hatching after 1 year No. (%)[4]
				artificial[2] No. (%)[4]	normal[3] No. (%)[4]	
Ro_1-Mierenbos	6	W	168	46 (27)	6 (4)	110 (65)
	12	B	167	71 (43)	3 (2)	134 (80)
Ro_3-C_{133}	8	Y	141	17 (12)	4 (3)	103 (73)
	12	B	173	76 (44)	7 (4)	105 (61)
Ro_5-Harmerz	6	W	145	30 (20)	7 (5)	128 (88)
	12	B	175	57 (33)	3 (2)	148 (85)
LSD (P<0.05)				5.1	2.5	3.3

[1] W=white, Y=yellow, B=brown.

[2] Halved cysts incubated in root diffusate.

[3] Whole cysts incubated in root diffusate.

[4] Percentage of average number of eggs per cysts.

Discussion

The artificial hatching procedure described here enables the propagation of three to five generations a year in pots and five to six generations a year in Petri dishes, which considerably facilitates genetic analysis of potato cyst nematodes. When females are reared in Petri dishes (Mugniery and Person, 1976; Mugniery, 1982) controlled matings can be made and rapid analysis of the progeny is possible. Furthermore, this system allows rearing large quantities of a population or inbred line of interest in a very short time. For instance, artificially hatched larvae from population Ro_3-C_{133} gave an average multiplication factor of 16.4 in pots, which results, taking five generations a year, in an overall multiplication factor of 1.2×10^6. Such an approach is desirable in studying the inheritance of virulence of pathotypes of potato cyst nematodes and in producing populations which are homozygous for (a)virulence.

In Petri dishes the number of successful inoculations is high; 70% of the larvae developed into females. This high percentage is in part the result of favourable conditions in Petri dishes. Only two second-stage larvae were inoculated per root tip, which minimizes the competition for food. Our findings support the theory that sex determination is epigenic (Mugniery and Fayet, 1984). During the course of this study males were hardly observed. However, in genetic studies using controlled matings single larva inoculation per Petri dish is recommended to avoid contamination with developed males.

That the remaining 30% of the second-stage larvae did not reach maturity may be the result of various factors. For instance, we observed that larvae left the inoculation site, started wandering through the Petri dishes and starved. Also some larvae failed to develop to maturity after penetration.

From the figures in Table 1 it can be calculated that 63% of the developed females were fertilized. This percentage is high compared with 45% obtained by Green et al. (1970). This disparity may in part be explained by differences in the methods.

Artificial hatching of second-stage larvae reared in Petri dishes and pots did not affect the viability and fecundity. The number of

successful inoculations and fertilizations in Petri dish experiments and the multiplication factor in pot experiments did not decline in subsequent generations.

Our results show that the water regime in the cysts and cutting the cyst wall is essential in bypassing the natural diapause. When cysts were left to desiccate the number of artificially hatchable larvae was strongly reduced. Similarly the hatchability dropped to about 3% when whole wet brown cysts, not being cut, were placed in hatching agent.

It is noted that the storage for a year favours the hatchability. The number of hatched larvae was 75% on an average of the eggs for one year air-dry stored cysts of the pot experiment, whereas this figure was 40% for new moist cysts when artificial hatching was applied. However, the number of larvae obtained by the artificial hatching procedure accomodates the scope of genetic research.

References

Clark, A.J. & Perry, R.N. (1977). Hatching of cyst-nematodes. Nematologica, 23: 350-368.

Cunningham, P.C. (1960). An investigation of winter dormancy in Heterodera rostochiensis. Scient. Proc. R. Dubl. Soc., 1: 1-4.

Ellenby, C. & Smith, L. (1967). Emergence of larvae from the new cysts of the potato-root eelworm, Heterodera rostochiensis. Nematologica, 13: 273-278.

Green, C.D., Greet, D.N. & Jones, F.G.W. (1970). The influence of multiple mating on the reproduction and genetics of Heterodera rostochiensis and H. schachtii. Nematologica, 16: 309-326.

Guile, C.T. (1967). On cyst colour changes, bionomics and distribution of potato cyst-eelworm (Heterodera rostochiensis Woll.) pathotypes in the East Midlands. Ann. appl. Biol., 60: 411-419.

Kort, J. (1960). A technique for the extraction of Heterodera cysts from wet soil and for the estimation of their eggs and larval content. Versl. Meded. Plziektenk. Dienst, Wageningen, 233: 3-7.

Kort, J., Ross, H., Rumpenhorst, H.J. & Stone, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst-nematodes Globodera rostochiensis and G. pallida. Nematologica, 23: 333-339.

McKenna, L.A. & Winslow, R.D. (1972). Differences in hatch and development rates of potato cyst nematode pathotypes. Ann. appl. Biol., 71: 274-278.

Mugniery, D. (1982). A method for screening potatoes for resistances to Globodera ssp. under laboratory conditions. In: Hooker, W.J. (Ed.). Research for the Potato in the Year 2000, CIP: 137-138.

Mugniery, D. & Fayet, G. (1984). Détermination du sexe de Globodera rostochiensis Woll. et influence des niveaux d'infestation sur la pénétration, le développement et le sexe de ce nématode. Revue de Nématologie, 7: 233-238.

Mugniery, D. & Person, F. (1976). Méthode d'élevage de quelques nématodes à kystes du genre Heterodera. Sci. Agron., Rennes, 217-220.

Oostenbrink, M. (1960). Estimating nematode populations by some selected methods. In: Sasser, J.N. & Jenkins, W.R. (Eds.). Nematology, Chapel Hill, Univ. of North Carolina Press: 85-102.

Rode, H. (1971). Der Einfluss verschiedener Temperaturen und Wechselreize auf das Schlüpfen von Larven des Kartoffelnematoden. Pedobiolog., 11: 143-158.

Shepherd, A.M. & Cox, P.M. (1967). Observations on periodicity of hatching of eggs of the potato cyst nematode, Heterodera rostochiensis Woll. Ann. appl. Biol., 60: 143-150.

CHAPTER II

ASSESSING INTRA-SPECIFIC VARIATIONS IN VIRULENCE IN *GLOBODERA ROSTOCHIENSIS* AND *G. PALLIDA*

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Abstract

The accuracy of the method of Mugniery and Person (1976) to rear cysts on roots of sprouts of potato in Petri dishes to estimate the percentage of (a)virulent phenotypes in populations of pathotypes of potato cyst nematodes, was investigated. Percentages of (a)virulent phenotypes were assessed for several pathotypes of *Globodera rostochiensis* and *G. pallida*. Frequencies of virulent phenotype values were compared with P_f/P_i assessments.

Introduction

Currently 8 pathotypes are recognized in Europe by the international pathotype scheme, five within *G. rostochiensis* (Woll.) Behrens and three within *G. pallida* (Stone) Behrens (Kort *et al.*, 1977). Pathotypes are defined by their multiplication rates (P_f/P_i-values) on a number of clones with different genes for resistance. Usually P_f/P_i-values are determined in pot experiments by inoculating approximately 25 full cysts (P_i) and assessing the number of newly developed cysts (P_f). Populations are classified as virulent or avirulent for a

differential if P_f/P_i-values are respectively > 1 or ≤ 1. This implies that populations of pathotypes classified as identical do not necessarily have the same rates of multiplication on these differentials. P_f/P_i-values ranged from 1 to 67 on a susceptible clone and from 0 to 1 on a resistant clone (Kort et al., 1977). Pathotype classification using current schemes is therefore inadequate to describe the virulence characteristics of a population. To characterize populations properly, it is recommended that estimates of the virulent phenotypes are also included (Bakker et al., 1988).

Multiplication rates alone on a differential clone are not an appropriate measure for the level of virulence in a population. P_f/P_i-values are density dependent and influenced by differences in hatching (Forrest & Phillips, 1984; Rawsthorne & Brodie, 1986). A more accurate approach to estimate the frequencies of virulent genotypes is to express the P_f/P_i on a differential as a percentage of the P_f/P_i on a general susceptible host. Unfortunately, near-isogenic lines with and without genes for resistance do not exist, implying that rates of multiplication are also influenced by genes other than those for resistance. Dale and Phillips (1985), for instance, reported variations in degree of susceptibility in cultivars of Solanum tuberosum ssp. tuberosum. These variations may be the result of factors such as differences between clones in morphology of root systems, production of hatching agents or other secondary plant metabolites. In view of these considerations it is evident that percentages are not ideal measures for the number of virulent genotypes in a population because these genotypes may multiply at different rates on a differential and susceptible clone. Moreover P_f/P_i-values are extremely sensitive to environmental factors and often result in large variation between and within experiments (Phillips, 1985).

Problems associated with pot experiments and the lack of near-isogenic potato lines can be minimized with the method developed by Mugniery and Person (1976) by rearing cysts on roots of sprouts in Petri dishes. This method offers better opportunities to control environmental conditions, excludes variations in factors such as differences in rates of root growth and hatching, and allows

standardization of inoculum densities per root tip.

In this report we evaluated the merits of the method of Mugniery and Person (1976) in estimating the number of virulent phenotypes in populations of pathotypes and compared these figures with results obtained from pot experiments.

Materials and methods

Experiments were carried out on roots of potato cultivars on water agar in Petri dishes and in pot experiments. Techniques for Petri dish experiments were those of Mugniery and Person (1976) and Mugniery (1982a). Experiments in pots were done in 700 ml clay pots with a loamy sandy soil in a controlled environment with 16 h light and at 18 °C. The inoculum density was 25 cysts per pot unless otherwise stated.

The differential clones Solanum tuberosum ssp. tuberosum L., S. tuberosum ssp. andigena Juz. and Buk., S. vernei Bittm. and Wittm. hybrid 58.1642/4 and S.vernei hybrid 62.33.3 (Kort et al., 1977) were replaced by the commercially available cultivars 'Eigenheimer', 'Saturna', 'Mara' and 'Darwina' respectively.

Population Ro_5-Harmerz (Ro_5-H) and Pa_3-Frenswegen were supplied by Dr. H.J. Rumpenhorst, Muenster, FRG, population Pa_2-HPL_1 by Ir. A. Mulder, Hilbrands Laboratorium, Assen, the Netherlands, and other populations by Ir. C. Miller, Plant Protection Service, Wageningen, the Netherlands. Pathotype designation and codes refer to the original collections.

To ensure representative samples of the populations, as a standard, 100 cysts of a population were used for hatching. Normal and artificial hatching of the larvae (Janssen et al., 1987) was carried out with root diffusate obtained from S. tuberosum ssp. tuberosum cultivar 'Bintje'.

In Petri dish experiments virulent phenotypes in populations of pathotypes were calculated by expressing the number of females that developed on a differential clone as a percentage of females that developed on a susceptible clone. In pot experiments estimates of virulent phenotypes were expressed as the multiplication rates on a differential clone relative to the P_f/P_i on a susceptible clone.

Four sets of experiments were carried out:

Experiment 1 -Petri dish/cultivar 'Eigenheimer'/Ro_1-Mierenbos. Five replicates (200 larvae/replicate); inoculum density: one to ten larvae/root tip.

Experiment 2 -Petri dish/ cultivar 'Eigenheimer'/Ro_5-Harmerz and cultivar 'Saturna'/Ro_3-C_{133}. Five to eleven replicates; inoculum density: two larvae/root tip; inoculum size: 25 to 500 root tips.

Experiment 3 -Petri dish/cultivar 'Eigenheimer' and cultivar 'Saturna'/five Ro_3 populations. Ten replicates; inoculum density: two larvae/root tip; inoculum size: 50 and 75 root tips for respectively cultivar 'Eigenheimer' and cultivar 'Saturna'.

-Pots/ cultivar 'Eigenheimer' and cultivar 'Saturna'/five Ro_3 populations. Ten replicates; inoculum: 25 cysts/pot of 700 ml.

Experiment 4 -Petri dish/cultivars 'Saturna', 'Mara' and 'Darwina'/three G. rostochiensis and three G. pallida populations. Inoculum density: two larvae/root tip; inoculum size: 100 and 500 root tips for respectively susceptible and resistant combinations.

-Pots/cultivar 'Saturna' and cultivar 'Darwina'/Ro_1-Mierenbos. Ten replicates; inoculum: 250 cysts/pot of 1.5 l.

Results

Inoculum density (experiment 1) and inoculum size (experiment 2)

Numbers of larvae inoculated per root tip were negatively correlated with those that developed into females (Table 1). When only one larva was inoculated per root tip 75% became female. Increasing the density to two or three larvae per root tip did not significantly affect the development into females. Upon inoculation with 5 or 10 larvae per root tip these percentages significantly dropped to 59% and 48% respectively. Inoculation with one larva per root tip would be optimal for estimating percentage of virulent phenotypes in populations of pathotypes, but is laborious. Therefore, as a standard we inoculated two larvae per root tip. Increasing the number of root tips inoculated per replicate (inoculum size) decreases the standard deviation and

Table 1. Decrease of the numbers of females of Ro_1-Mierenbos on the susceptible cultivar 'Eigenheimer' by increasing the inoculation density per root tip[1].

Inoculation density per root tip	Number of root tips inoculated	Percentage of females	SD
1	200	75 a[2]	4.2
2	100	72 a	3.0
3	67	70 a	3.1
5	40	59 b	2.5
10	20	49 c	3.0

[1] Five replicates per treatment.

[2] Figures bearing the same letter are not significantly different (5% level).

Table 2. Decrease of the standard deviations by increasing the numbers of inoculated root tips in two combinations: Ro_5-Harmerz on cultivar 'Eigenheimer' and Ro_3-C_{133} on cultivar 'Saturna'. In these combinations 69.8% and 3.0% of the larvae developed into females respectively[1].

Number of root tips inoculated	Number of replicates	Ro_5-Harmerz Eigenheimer SD	Ro_3-C_{133} Saturna SD
25	11	6.6	----[2]
50	10	3.5	0,84
100	8	2.9	----[2]
300	5	---[2]	0.62
500	5	---[2]	0.39

[1] Two larvae were inoculated per root tip.

[2] Experiments not carried out.

favours the accuracy of the test (Table 2). The values of the standard deviations are in accordance with those obtained from the binomial distribution.

The accuracy of the calculated frequencies of virulent phenotypes is illustrated with the low standard deviations (SD), ranging from 0.23 to 1.63, obtained with five populations of pathotype Ro_3 on cultivar 'Saturna' (Table 3).

Comparison of Petri dish and pot experiments in assessing percentage of virulent phenotypes (experiment 3)

Table 3 also compares the accuracy of the method of Mugniery and Person with pot tests in estimating numbers of virulent phenotypes in five populations of pathotype Ro_3. The estimates of virulent phenotypes with the Petri dish are consistently higher than those from

Table 3. Fraction of virulent phenotypes ($\overline{vp}$) with SD and CV of five RO_3-populations for cultivar 'Saturna' calculated as percentages of the number of females that developed on the susceptible cultivar 'Eigenheimer'.

		Ro_3-populations				
		C_{129}	C_{176}	C_{150}	C_{156}	C_{163}
Petri dish	$\overline{vp}$	30.19[2]	1.54[2]	14.56[2]	23.44[2]	27.79[2]
experiment[1]	SD	1.51	0.23	1.21	1.51	1.63
	CV%	5.00	14.94	8.31	6.44	5.87
Pot experiment[1]	$\overline{vp}$	8.09	1.35	10.46	9.97	13.06
	SD	4.75	0.98	4.92	2.87	6.48
	CV%	58.71	72.79	47.04	28.79	49.61

[1] 10 replicates.

[2] Inoculum size of 50 root tips for cultivar 'Eigenheimer' and 75 for cultivar 'Saturna'; two larvae inoculated per root tip.

pot experiments. The standard deviations are low in experiments in Petri dishes and higher in pot experiments. Similarly the coefficients of variation calculated from Petri dish experiments are low ranging from 5.00 to 14.94, and are considerably higher in pot experiments varying from 28.79 to 72.79.

Frequencies of virulent phenotypes of populations of pathotypes (experiment 4)

A number of populations of pathotypes were screened for their frequencies of virulent phenotypes on three differentials using the Petri dish method and inoculating two larvae per root tip (Table 4). The number of virulent phenotypes for the differential clones vary per

Table 4. Fractions of virulent phenotypes on three differentials in six populations of pathotypes calculated as percentages of the number of females that developed on the susceptible cultivar 'Eigenheimer'. The SD is given in brackets.

Pathotype population	Differentials (Cultivars)		
	S.tuberosum ssp. *andigena* ('Saturna')	*S.vernei* hybr. 58.1642/4 ('Mara')	*S.vernei* hybr. 62.33.3 ('Darwina')
Ro_1-Mierenbos	-----[1]	0.24 (0.04)	----[1]
Ro_3-C_{133}	5.0 (0.4)	0.5 (0.1)	0
Ro_5-Harmerz	84.6 (3.7)	68.5 (3.4)	15.9 (1.7)
Pa_2-HPL_1	102.9 (5.1)	64.3 (3.2)	1.5 (0.1)
Pa_3-E_{1202}	100.0 (5.4)	101.9 (4.9)	6.7 (0.7)
Pa_3-Frenswegen	-----[2]	-----[2]	5.7 (0.6)

[1] Percentages of virulent phenotypes according to pot tests < 0.10.

[2] Experiments not carried out.

population. For example, populations Ro_3-C_{133}, Ro_5-H, Pa_2-HPL_1 and Pa_3-E_{1202} (all four of pathotypes able to overcome the resistance of the gene H_1 from S. tuberosum ssp. andigena (Toxopeus & Huijsman, 1953) in cultivar 'Saturna' differ considerably in their frequencies of virulent phenotypes. Population Ro_3-C_{133} has only 5% virulent phenotypes whereas these percentages range from 84 to 100 in the other populations tested.

Population Ro_1-Mierenbos has very low frequencies of virulent phenotypes. Only 0.24 % is able to develop on the differential S. vernei 58.1642/4. In our Petri dish experiments, no virulent phenotypes could be detected for the differentials S. tuberosum ssp. andigena and S. vernei 62.33.3. However, in a pot experiment carried out in 10 replicates with an inoculum size of at least 250 cysts per 1.5 l pot a few females developed, indicating that frequencies of virulent phenotypes for both differentials are less than 0.1 %.

The differential S. vernei 62.33.3 completely blocks the development of individuals of population Ro_3-C_{133} as no cysts were found in Petri dish or pot tests.

Discussion

The method of Mugniery and Person (1976) has several advantages compared to pot tests or experiments in closed containers (Foot, 1977; Phillips et al., 1980) in estimating virulent phenotypes in populations. Petri dish experiments allow better controlled environmental conditions, standardization of inoculum densities per root tip and they circumvent differences in hatching (Forrest & Phillips, 1984; Rawsthorne & Brodie, 1986) and morphology of root systems.

The accuracy of the Petri dish method is clearly demonstrated by the low standard deviation and coefficients of variation (Table 2, 3). On the basis of standard deviations we used 100 root tips (200 larvae) in susceptible and 500 root tips (1000 larvae) in resistant combinations. In assessments of extremely low numbers of virulent phenotypes, more root tips have to be inoculated in resistant combinations (e.g. Table 4).

The observation that the Ro_3 population scored considerably lower when tested in pot experiments than in Petri dishes (Table 3) is probably due to the differences in the root systems in pots. The root system of the cultivar 'Saturna' with the H_1 resistance gene was consistently less developed than that of the susceptible cultivar 'Eigenheimer'. This disparity may differentially affect multiplication rates by factors not related to the H_1 gene such as hatching, host finding or inoculum densities per root tip. These effects are evidently neutralized in Petri dish experiments. Therefore, figures obtained from Petri dish experiments are a more accurate estimate of the virulent genotypes. For that reason the advantages of the Petri dish method also makes it a reliable tool for analyzing fitness expressions in subsequent generations.

Increasing the number of larvae per root tip significantly decreases the production of females (Table 1). These observations confirm those of Mugniery and Fayet (1981) and give support to the idea that sex determination in potato cyst nematodes is epigenetic and influenced by factors such as competition or physiological conditions (Trudgill, 1967; Mugniery, 1982b, 1985; Mugniery & Fayet, 1981, 1984). Effects of epigenetic factors on the assessments of virulent phenotypes in populations of pathotypes were minimized by inoculating two larvae per root tip and by selecting uniform root tips. The numbers of females that developed on cultivar 'Eigenheimer' were in accordance with the maximum female production obtained by Mugniery (1985).

The estimates of virulent phenotypes in five Ro_3 populations (Table 3) illustrate the shortcomings of the international pathotype scheme of Kort *et al.* (1977). The percentages of virulent individuals in these populations showed marked variations. Although such results are expected from panmictic populations these interpopulational variations are often neglected. Until now the pathotype scheme was accepted as a proper classification in elucidating virulence and resistance mechanisms at the cellular and molecular levels (*e.g.* Bakker & Gommers, 1982; Fox & Atkinson, 1984; Greet & Firth, 1977; Ohms & Heinicke, 1985; Stegemann *et al.*, 1982; Wharton *et al.*, 1983). For this reason much of the existing information on pathotypes and resistance is unreliable and needs to be reevaluated (Triantaphyllou,

1987).

Resistance in potato is mainly derived from S. tuberosum ssp. andigena and S. vernei and is mediated by pathotype specific genes. Jones et al. (1967) suggested that the resistance conferred by the H_1 gene and virulence in the nematode are operating on the basis of a gene-for-gene relationship (Flor, 1956). For S. vernei hybrids this suggestion can not be made, as yet, because of the polygenic nature of the resistance. Larvae that are able to develop into females on clones having the H_1 gene are thought to be double recessives (aa) whilst those developing into males can have any genetic constitution (AA, Aa or aa). A major difficulty is to relate the virulent phenotypes in populations to the number of virulent genotypes. Virulent phenotypes are calculated from Petri dish experiments by expressing the number of females that appeared on the roots of the differential as a percentage of the numbers of females on the susceptible cultivar. This percentage is only an appropriate measure for the number of virulent genotypes if genes other than the H_1 gene do not affect the development of double recessive larvae i.e. the fraction of virulent individuals that develops on the differential is the same as on the susceptible clone.

The correctness of this approach can be proven in two ways. First by developing lines with 100 % double recessive individuals. In this case the numbers of females that develop on a H_1 resistant clone can be used as a reference to calculate virulent phenotypes instead of those that developed on a susceptible clone. The alternative is the production of near-isogenic lines with and without the H_1 gene and to use - as was done in this study - the susceptible combination as reference.

Research on biochemistry and physiology of host parasite interaction would benefit from the availability of nematode populations with 100 % virulent individuals on one hand and with 0 % virulent individuals on the other. As shown in Table 3 and Table 4 this condition is not matched with either of the two species. Only G. pallida population Pa_2-HPL_1 and Pa_3-E_{2102} seem to be fixed for certain virulence alleles but no G. pallida populations are available which are fixed for the corresponding avirulence alleles. These data demonstrate the need for lines of potato cyst nematodes with 100 % (a)virulence for a given

gene for resistance or resistance complex. Future research is aimed at the selection of lines with defined genetic constitution by controlled single matings in Petri dishes.

References

Bakker, J., Schots, A., Bouwman-Smits, L. & Gommers, F.J. (1988). Species-specific and thermostable proteins from second-stage larvae of Globodera rostochiensis and G. pallida. Phytopathology, 78: 300-305.

Bakker, J. & Gommers, F.J. (1982). Differentation of the potato cyst nematodes Globodera rostochiensis and G. pallida and of two G. rostochiensis pathotypes by means of two-dimensional electrophoresis. Proc. K. Ned. Akad. Wet., C85: 309-314.

Dale, M.F.B. & Phillips, M.S. (1985). Variation for the degree of susceptibility to the potato cyst nematode (Globodera pallida) with Solanum tuberosum ssp. tuberosum. Potato Res., 28: 55-64.

Flor, H.H. (1956). The complementary genetic systems in flax and flax rust. Adv. Genet., 8: 29-54.

Foot, M.A. (1977). Laboratory rearing of potato cyst nematode; a method suitable for pathotyping and biological studies. N.Z. J. Zool., 4: 183-186.

Forrest, J.M.S. & Phillips, M.S. (1984). The effect of Solanum tuberosum x S. vernei hybrids on hatching of the potato cyst nematode Globodera pallida. Ann. appl. Biol., 104: 521-526.

Fox, P.C. & Atkinson, H.J. (1984). Isoelectric focusing of general protein and specific enzymes from pathotypes of Globodera rostochiensis and G. pallida. Parasitology, 88: 131-139.

Greet, D.N. & Firth, J. (1977). Influence of host plant on electrophoretic protein patterns of some round-cyst nematode females and use of larvae to obtain less ambiguous results. Nematologica, 23: 411-415.

Janssen, R., Bakker, J. & Gommers, F.J. (1987). Circumventing the diapause of potato cyst nematodes. Neth. J. Pl. Path., 93: 107-113.

Jones, F.G.W., Parrott, D.M. & Ross, G.S.J. (1967). The population genetics of the potato cyst-nematode, Heterodera rostochiensis: mathematical models to simulate the effects of growing eelworm-resistant potatoes bred from Solanum tuberosum ssp. andigena. Ann. appl. Biol., 60: 151-171.

Kort, J., Ross, H., Rumpenhorst, H.J. & Stone, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst-nematodes Globodera rostochiensis and G. pallida. Nematologica, 23: 333-339.

Mugniery, D. (1982a). A method for screening potatoes for resistance to Globodera spp. under laboratory conditions. In: Hooker, W.J. (Ed.). Research for the Potato in the Year 2000, CIP: 137-138.

Mugniery, D. (1982b). Influence de l'hôte sur le developpement et l'expression du sexe chez un nématode phytoparasite à déterminisme sexuel épigénique, Globodera pallida Stone. C. R. Hebd. Séanc Acad. Sci., Paris, 294, Sér. III: 49-52.

Mugniery, D. (1985). Résistance des solanacées à Globodera et influence de certains facteurs sur les résultats des tests de résistance. EPPO Bull., 15: 199-206.

Mugniery, D. & Fayet, G. (1981). Détermination du sexe chez Globodera pallida Stone. Revue Nématol., 4: 41-45.

Mugniery, D. & Fayet, G. (1984). Détermination du sexe de Globodera rostochiensis Woll. et influence des niveaux d'infestation sur la pénétration, le développement et le sexe de ce nématode. Revue Nématol., 7: 233-238.

Mugniery, D. & Person, F. (1976). Méthode d'elevage de quelques nématodes à kystes du genre Heterodera. Sci. Agron., Rennes, 217-220.

Ohms, J.P. & Heinicke, D.H.K. (1985). Pathotypen des Kartoffelnematoden. II. Bestimmung der Rassen von Globodera rostochiensis durch die Mikro-2D-Elektrophorese von Einzelzysten. Z. PflKrankh. PflSchutz, 92: 225-232.

Phillips, M.S. (1985). Environmental differences and their effect on the assessment of quantitative resistance to potato cyst nematodes. EPPO Bull., 15: 179-183.

Phillips, M.S., Forrest, J.M.S. & Wilson, L.A. (1980). Screening for resistance to potato cyst nematode using closed containers. Ann. app. Biol., 96: 317-322.

Rawsthorne, D. & Brodie, B.B. (1986). Relationship between root growth of potato, root diffusate production, and hatching of Globodera rostochiensis. J. Nematol., 18: 379-384.

Stegemann, H., Francksen, H. & Rumpenhorst, H.J. (1982). Differenzierung der Nematoden Globodera rostochiensis und G. pallida durch ein- und zweidimenzionale Elektrophoresen ihrer Proteine. Nachrichtenb. Deut. Pflanzenschutzd., 34: 3-7.

Toxopeus, H.J. & Huijsman, C.A. (1953). Breeding for resistance to potato root eelworm, I. Preliminary data concerning the inheritance and nature of resistance. Euphytica, 2: 180-186.

Triantaphyllou, A.C. (1987). Genetics of nematode parasitism on plants. In: Veech, J.A. & Dickson, D.W. (Eds.). Vistas on Nematology, Society of Nematologists, Inc., Maryland: 354-363.

Trudgill, D.L. (1967). The effect of environment on sex determination in Heterodera rostochiensis. Nematologica, 13: 263-272.

Wharton, R.J., Storey, R.M.J. & Fox, P.C. (1983). The potential of some immunochemical and biochemical approaches to the taxonomy of potato cyst nematodes. In: Stone, A.R., Platt, H.M. & Khalil, L.K. (Eds.). Concepts in Nematode Systematics, Academic Press, Inc., New York: 235-248.

CHAPTER III

SELECTION OF LINES OF *GLOBODERA ROSTOCHIENSIS* VIRULENT AND AVIRULENT TO THE H_1 RESISTANCE GENE IN *SOLANUM TUBEROSUM* SSP. *ANDIGENA* CPC 1673

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Abstract

Virulent and avirulent lines of *Globodera rostochiensis* for the H_1 resistance gene in *Solanum tuberosum* ssp. *andigena* CPC 1673 were obtained with controlled single matings. The selection of virulent and avirulent lines was less straightforward than expected from the gene-for-gene theory. First, the number of lines with intermediate levels of virulence was larger than expected and, second, in many of these lines the levels of virulence did not agree with the genetic interpretation according to the theory.

Introduction

In the international pathotype scheme the pathotypes of *Globodera rostochiensis* (Ro_1-Ro_5) and *Globodera pallida* (Pa_1-Pa_3) are delineated by their reproduction pattern on sets of differentials (Canto Saenz & de Scurrah, 1977; Kort *et al.*, 1977). The pathotypes were already present in Europe before resistant cultivars were grown. Variations in virulence are predominantly the result of four processes: 1) the genetic structures of the initial populations introduced from South

America, 2) random genetic drift and 3) gene flow (Bakker, 1987). Since the late sixties the frequencies of alleles for virulence have also been affected by selection caused by the cultivation of resistant potato cultivars.

Estimates of the virulent phenotypes indicated that these four processes have not led to field populations fixed for avirulence or virulence alleles for the H_1 resistance gene of S. tuberosum ssp. andigena CPC 1673 (Janssen et al., 1990a). The lack of such monomorphic populations hampers research aimed at unravelling the genetics of virulence.

Earlier genetic research (Parrott ,1981) to verify the theory of a gene-for-gene relationship (Howard, 1959; Jones & Parrott, 1965) did not result in conclusive data because the levels of virulence of the lines used had not been clearly defined. Initiatives to breed the appropriate virulent and avirulent lines have been discouraged by the laboriousness of the genetic analysis, the absence of an accurate test to assess levels of virulence, and unreliable techniques for controlled matings. We solved these difficulties by circumventing the diapause (Janssen et al., 1987) and by rearing females and cysts on roots of sprouts grown on agar plates (Janssen et al., 1990a; Mugniery and Person, 1976). With these techniques we were able to breed lines of G. rostochiensis either completely avirulent or highly virulent to host genotypes carrying the H_1 resistance gene.

Materials and methods

The population Ro_1-Mierenbos was used to select avirulent lines and the populations Ro_3-C_{133}, Ro_3-C_{129} and Ro_5-Harmerz were used to select virulent lines. The populations were supplied by the Plant Protection Service, Wageningen, the Netherlands, except for Ro_5-Harmerz which was obtained from Dr. H.J. Rumpenhorst, Münster, FRG. The cultivar 'Eigenheimer' was used as the universal susceptible clone. The differential Solanum tuberosum ssp. andigena CPC 1673 with the H_1 resistance gene was represented by the cultivar 'Saturna'.

Selection of virulent lines

Virulent lines for the H_1 gene were selected as follows. Adult females for controlled matings were reared in Petri dishes (9 ϕ cm) on roots of sprouts of cultivar 'Saturna' grown on water agar according to the method of Mugniery and Person (1976) and Mugniery (1982). To prevent inbreeding, only one larva per Petri dish was inoculated. Females were fertilized by placing the male on top of the gelatinous matrix of the female. Since it is generally accepted that resistance has no selective influence on the development of larvae into males (Jones et al., 1967), populations were in all cases reared on cultivar 'Eigenheimer' grown in pots to secure sufficient numbers of males.

These single crosses (F_1) were multiplied, full-sib mating, by inoculating one cyst per pot on cultivar 'Saturna' after artificial hatching (Janssen et al., 1987). Cysts were cut in halves and these halved cysts were inoculated by pouring the larvae (L_2) suspension in preformed holes in the soil when the plants were approximately 15 cm tall. Multiplication of the F_2 and F_3 progeny (lines) on cultivar 'Saturna' was done in a similar way by inoculating larval suspensions from 10 cysts or less per pot. The number of virulent phenotypes in the F_3 or F_4 was estimated by expressing the number of females that developed on the resistant cultivar 'Saturna' as a percentage of those that developed on the susceptible cultivar 'Eigenheimer'. These virulence tests were carried out in Petri dishes by inoculating two second stage L_2 per root tip (Janssen et al., 1990a). Two hundred L_2 were inoculated on each cultivar. To guarantee a random sample of L_2, 100 cysts were used for hatching. When less than 100 cysts were available for testing, the F_3 line was multiplied once more on cultivar 'Saturna'. The whole procedure was completed within an average period of 15 months: one generation in Petri dishes, three generations in pots, and the virulence test in Petri dishes again.

Selection of avirulent lines

The procedure for the selection of lines avirulent to the H_1 gene was similar, except that for all generations, the susceptible cultivar

'Eigenheimer' was used. Testing for virulence to cultivar 'Saturna' was carried out in pots (700 ml) instead of Petri dishes because of the higher detection level for low virulence frequencies in pots. Virulence levels were calculated by expressing the number of females that developed on cultivar 'Saturna' as a percentage of those that developed on cultivar 'Eigenheimer'. Due to the limited number of cysts, the inoculation density was not the same for the susceptible (P_i=10 cysts) and the resistent cultivar (P_i=40 cysts). This methodology resulted in an overestimation of the number of virulent phenotypes.

To study the virulence levels in the progeny of single male-female matings a sufficient number of cysts are required. For a monogenic inheritance of virulence as described by Jones et al. (1967) with three possible genotypes (AA, Aa, and aa), with the virulent genotype (aa) being present in 2% of the individuals and the population being in Hardy-Weinberg equilibrium, the frequency of the a allele (q) is 14% and of the A allele (p) 86%. This results in 24% and 74% of the Aa and AA genotype, respectively. The fraction of cysts with aa larvae will be $(0.24 + 0.02)^2 = 0.07$ and the fraction without aa larvae 0.93. The presence of virulent larvae in the hatching sample is assured when 40 cysts are taken since the chance of cysts without aa larvae is less than 5%. In this study we always used 40 or more cysts.

All pot experiments were carried out in clay pots (700 ml) with loamy sandy soil and a slow release N-P-K granulate fertilizer (Osmocote R) in a controlled environment room at 18 °C with 16 h light. Petri dishes covered with black plastic were stored under the same conditions.

To optimalize the recovery of wet cysts from wet soil, a Kort elutriator (Kort, 1960) was used at the maximum water stream of 4.5 l/min, and a special sieve (pore size 0.30 x 3.65 mm) was used to collect the cysts. For all experiments, males were reared in pots on the susceptible cultivar 'Eigenheimer' and recovered from the soil with an Oostenbrink elutriator (Oostenbrink, 1960).

Results

Selection of lines virulent to the H_1 gene

Population Ro_3-C_{133} and Ro_3-C_{129} had low levels of virulence. The virulence level was enhanced by rearing these populations on cultivar 'Saturna' in pots for several generations. After four generations the percentage of virulent phenotypes of population Ro_3-C_{129} increased from 30.1% to 50.8%. Controlled single crossings in Petri dishes resulted in 19 fertilized F_1 cysts. Unfortunately only one cyst multiplied to a F_2 containing 18 cysts; this was insufficient for a virulence test and these cysts were not used in further experiments. With the Ro_3-C_{133} population having 1.4% virulent phenotypes we were unable to select lines virulent to the H_1 gene because the population died out after three generations of multiplication in pots.

Table 1. Fraction of virulent phenotypes (vp%) of F_3 and F_4 Ro_5-Harmerz lines on cultivar 'Saturna', calculated as percentages of the number of females that developed on the susceptible cultivar 'Eigenheimer'.

	line no								
	4	7*	8	19	22*	23	34	47	52
vp%	93.1 b	91.7 bc	87.0 c	77.8 d	100.2 a	—[1]	—[1]	94.0 b	—[1]

* F_3 lines

[1] Virulence test not carried out

Lines with the same letter are not significantly different ($P < 0.05$)

Population Ro_5-Harmerz proved to be suitable for the selection of virulent lines. The initial number of virulent phenotypes in this population was 84.6 % (Janssen et al., 1990a). Inoculation of 579 root tips of the resistant cultivar 'Saturna' resulted in the development of 252 females. Mating these virgins with single males resulted in 164 fertilized females. Of these F_1-cysts 87.4% were found to reproduce on the cultivar 'Saturna' in pots. Nine F_2 lines were selected for multiplication. To obtain sufficient cysts for virulence tests seven of the nine F_3 lines were multiplied once more on the cultivar 'Saturna'. In the virulence tests one line (no.22) showed a percentage of virulent phenotypes of approximately 100% (Table 1).

Production of lines avirulent to the H_1 gene

Population Ro_1-Mierenbos was chosen because the initial number of virulent phenotypes in that population was low (<0.1%) (Janssen et al., 1990a). Inoculating 585 root tips with one larva each resulted in the development of 416 females of which 72.4% were sucessfully fertilized. Eighty-six F_1 cysts were multiplied in pots on cultivar 'Eigenheimer', and we obtained 65 F_3-lines. Nineteen lines were tested. Eleven lines produced no cysts on cultivar 'Saturna' (Table 2). Six of these eleven avirulent lines were tested again on cultivar 'Saturna', and only two of these lines produced no cysts in the second test (Table 3).

Table 2. Fraction of virulent phenotypes (vp%) of F_3 Ro_1-Mierenbos lines for cultivar 'Saturna', calculated as percentages of the number of females that developed on the susceptible cultivar 'Eigenheimer'.

	line no																		
	1	6	8	11	12	14	15	16	17	18	19	22	24	27	42	44	51	52	58
vp%	2.1	0	2.8	0	3.4	0	0	0	0.2	2.8	0	0.6	0	0	1.0	0	0.5	0	0

Table 3. Retest of 6 Rol-Mierenbos lines on virulent phenotypes (vp%) for cultivar 'Saturna'.

line no	6	11	14	19	24	27
vp %	0	0.03	0	0.12	0.04	0.08

Discussion

Jones et al. (1967) suggested that the gene-for-gene hypothesis is also applicable to G. rostochiensis and S. tuberosum ssp. andigena CPC 1673, and that genes for virulence (a) and avirulence (A) were alleles of a single locus, avirulence being the dominant allele. They assumed that this interaction between alleles is confined to larvae developing into females. Males developing on cultivars having the H_1 gene can have any genotype (AA, Aa, aa). If this hypothesis is correct the estimate of the fraction of virulent phenotypes corresponds with the number of homozygous recessive genotypes (aa). This also implies that the F_1 cysts from population Ro_5-Harmerz able to reproduce on cultivar 'Saturna' are the progeny of either an aa x aa or an aa x Aa cross. The first type of cross will result in lines with 100% virulent genotypes, the second cross, as can be calculated according to Jones et al. (1967), will result in F_1 lines having 50% virulent genotypes, while the F_2, F_3 and F_4, produced on cultivar 'Saturna', will have virulence percentages of 75, 88 and 94, respectively. Not all our data match these calculations. The percentages of virulent phenotypes (Table 1) in line 4, 7 and 47, presumed to be derived from an aa x Aa cross, and line 22, from an aa x aa cross, fit the calculated expectations, whereas the percentages of virulent phenotypes in line 8 and 19 are significantly lower than the expected 94%.

Conclusive evidence that the percentage of virulent genotypes in line 22 is 100 indeed cannot be inferred from our data. Since no near-isogenic lines with and without resistance are available, the estimates of the percentage of virulent phenotypes are influenced by

the choice of the potato cultivars because these were made by expressing the number of females that developed on the resistant cultivar relative to the number on the susceptible cultivar. In a parallel report (Janssen et al., 1990b) we showed, that line 22 is, indeed, homozygous for virulence.

Another disparity with the gene-for-gene theory as proposed by Jones et al. (1967), is that the number of lines having a virulence percentage of 100 is relatively low (Table 1). From the initial frequencies of the virulent phenotypes in population Ro_5-Harmerz, it is expected that 84.6% of the F_1 cysts able to reproduce on 'Saturna' were derived from an aa x aa cross and 14.6% of the F_1 cysts from an aa x Aa cross. The results show that of the nine lines tested only one line had a virulence level of approximately 100% (line 22). Statistically this outcome does not correspond with the expected percentage. From the Jones' theory, the chance of such a result is less than 5%. The results of attempts to select virulent lines from Ro_3-C_{133} and Ro_3-C_{129} are puzzling too. For example, the virulence percentages in population Ro_3-C_{129} increased after four generations of multiplication on cultivar 'Saturna' from 30.1% to 50.8%, whereas an increase to 94.4% was expected.

Comparable contradictions with the gene-for-gene theory are also observed with the selection of avirulent lines from Ro_1-Mierenbos. First, only 57.9% of the lines produced no cysts on cultivar 'Saturna' (Table 2). Retesting 6 F_3 lines showed that this percentage was actually lower (Table 3). As can be calculated from the initial number of virulent phenotypes in population Ro_1-Mierenbos, the expected percentage of avirulent lines was 87.9%. Second, the lines producing cysts on cultivar 'Saturna' had virulence percentages ranging from 0.03 to 3.4 (Table 2 and 3), values too low to be explained by Jones' theory. According to the theory the lowest intermediate virulence percentage is 6, for lines derived from an AA x Aa cross. A possible explanation for the low virulence percentages observed, not in contradiction with Jones' theory, is that these lines are derived from an AA x AA cross and that the resistance mechanism conferred by the H_1 gene is not absolute. Definite proof that the females developed on cultivar Saturna are escapers and indeed avirulent (AA) can only be

obtained by crossing with double recessive males and by studying the virulence characteristics of the progenies.

Several studies have been aimed at studying the genetics of virulence towards the H_1 gene (Parrott & Berry, 1973; Parrott, 1981). Although no definitive proof was obtained for a gene-for-gene relationship, most of the data were in reasonable agreement with the calculated values (Jones et al., 1981). However, our data show that the selection of virulent and avirulent lines is less straightforward than expected from a gene-for-gene relationship. Evidently our data do not exclude such a relationship. Conclusive evidence to accept or reject the hypothesis can only be obtained by making crosses of the virulent and avirulent lines and studying the Mendelian behaviour of the virulence characters.

References

Bakker, J. (1987). Protein Variation in Cyst Nematodes. Ph.D. Thesis. Univ. of Agriculture, Wageningen, The Netherlands, 159 pp.

Canto Saenz, M. & de Scurrah, M.M. (1977). Races of potato cyst nematode in the Andean region and a new system of classification. Nematologica, 23: 340-349.

Howard, H.W. (1959). Biotypes of potato root eelworm in Great Britain. Tagber. Dt. Akad. Landw-Wiss. Berl., 20: 95-106.

Janssen, R., Bakker, J. & Gommers, F.J. (1987). Circumventing the diapause of potato cyst nematodes. Neth. J. Pl. Path., 93: 107-113.

Janssen, R., Bakker, J. & Gommers, F.J. (1990 a). Assessing intraspecific variations in virulence in Globodera rostochiensis and G. pallida. Revue Nématol., 13: in press.

Janssen, R., Bakker, J. & Gommers, F.J. (1990 b). Mendelian proof for a gene-for-gene relationship between virulence of Globodera rostochiensis and the H_1 resistance gene in Solanum tuberosum ssp. andigena CPC 1673. Revue Nématol., 13: accepted.

Jones, F.G.W. & Parrott, D.M. (1965). The genetic relationships of pathotypes of Heterodera rostochiensis Woll. which reproduce on hybrid potatoes with genes for resistance. Ann. appl. Biol., 56: 27-36.

Jones, F.G.W., Parrott, D.M. & Perry, J.N. (1981). The gene-for-gene relationship and its significance for potato cyst nematodes and their solanaceous hosts. In: Zuckerman, B.M. & Rohde, R.A. (Eds.). Plant Parasitic Nematodes, Vol. III, Academic Press, Inc., New York: 23-36.

Jones, F.G.W., Parrott, D.M. & Ross, G.S.J. (1967). The population genetics of the potato cyst-nematode, Heterodera rostochiensis: mathematical models to simulate the effects of growing eelworm-resistant potatoes bred from Solanum tuberosum ssp. andigena. Ann. appl. Biol., 60: 151-171.

Kort, J. (1960). A technique for the extraction of Heterodera cysts from wet soil and for the estimation of their eggs and larval content. Versl. Meded. Pziektenk. Dienst, Wageningen, 233: 3-7.

Kort, J., Ross, H., Rumpenhorst, H.J. & Stone, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst-nematodes Globodera rostochiensis and G. pallida. Nematologica, 23: 333-339.

Mugniery, D. (1982). A methode for screening potatoes for resistance to Globodera ssp. under laboratory conditions. In: Hooker, W.J. (Ed.). Research for the Potato in the Year 2000, CIP: 137-138.

Mugniery, D. & Person, F. (1976). Méthode d'élevage de quelques nématodes à kystes du genre Heterodera. Sci. Agron., Rennes, 217-220.

Oostenbrink, M. (1960). Estimating nematode populations by some selected methods. In: Sasser, J.N. & Jenkins, W.R. (Eds.). Nematology, Chapel Hill, Univ. of North Carolina Press: 85-102.

Parrott, D.M. (1981). Evidence for gene-for-gene relationships between resistance gene H_1 from Solanum tuberosum ssp. andigena and a gene in Globodera rostochiensis, and between H_2 from S. multidissectum and a gene in G. pallida. Nematologica, 27: 372-384.

Parrott, D.M. & Berry, M.M (1973). Selection of Dutch pathotypes of potato cyst-nematodes on resistant potatoes. Rothamsted Exp. Stn. Rep., Part 1: 152-155.

CHAPTER IV

MENDELIAN PROOF FOR A GENE-FOR-GENE RELATIONSHIP BETWEEN VIRULENCE OF *GLOBODERA ROSTOCHIENSIS* AND THE H_1 RESISTANCE GENE IN *SOLANUM TUBEROSUM* SSP. *ANDIGENA* CPC 1673

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Abstract

A virulent and an avirulent inbred line of *G. rostochiensis* were crossed to determine the genetics of virulence to the resistance gene H_1 of *Solanum tuberosum* ssp. *andigena* CPC 1673. The 3:1 segregation in avirulent and virulent larvae of the F_2 generation, obtained by selfing the F_1, showed that virulence to the H_1 gene is controlled by a single major recessive gene. The virulence percentages of the F_1 generations agreed with this finding. Reciprocal crosses showed no evidence of sex-linked inheritance of virulence.

Introduction

Breeding of potato varieties with resistance against potato cyst nematodes (*e.g.* Toxopeus & Huijsman, 1952, 1953; Huijsman, 1955) was initiated in the fifties with the discovery of the H_1 resistance gene in *Solanum tuberosum* ssp. *andigena* CPC 1673 by Ellenby (1952). The monogenetic inheritance resulted in successful breeding programmes which stimulated the search for more sources of resistance. Major

genes for resistance were identified in S. multidissectum: H_2 (Dunnett, 1961), S. tuberosum ssp. andigena CPC 2775: H_3 (Howard et al., 1970), S. kurtzianum: A,B (Huijsman, 1960) and S. spegazzinii: Fa,Fb (Ross, 1962). Resistance in S. vernei (e.g. Goffart & Ross, 1954; Huijsman, 1974; Huijsman & Lamberts, 1972; Kort et al., 1972; Ross, 1969), S. spegazzinii and S. oplocense (Ross, 1972) seemed to be caused by a complex of major and minor genes. A number of these sources of resistance, including S. vernei, are used in the international pathotype test assortment. With this assortment, five pathotypes of G. rostochiensis and three pathotypes of G. pallida can be recognized on basis of multiplication rates (Kort et al., 1977).

The variations in the virulence spectra of potato cyst nematode populations show similarities with host-parasite systems in which resistance and virulence operate in a gene-for-gene relationship. Howard (1959) and Jones & Parrott (1965) suggested that, similar to the relationship in Linum usitatissimum-Melampsora lini (Flor, 1956), the interaction between G. rostochiensis and the H_1 gene of S. tuberosum ssp. andigena CPC 1673 is controlled by a gene-for-gene mechanism. Females developing on a resistant clone are thought to be homozygous recessive; males can have any genetic constitution: AA, Aa or aa (Jones et al., 1967). Studies supporting this hypothesis (Parrott & Berry, 1974; Parrott, 1981), lacked the experimental and numerical base necessary for a formal proof according to Flor's criterion (Sidhu, 1975). They were based on mass matings between groups of males and females from field populations. The exact numbers of virulent individuals in these populations were unknown, sib matings may have occurred, and the testing of the relative numbers of female progeny on hosts with and without resistance was subject to large errors (Jones et al., 1981).

In this study we have avoided the difficulties associated with genetic studies on potato cyst nematodes by using inbred lines (Janssen et al., 1990b), controlled single matings and an improved technique to assess the number of virulent phenotypes in the progenies (Janssen et al., 1990a).

Materials and methods

Analyses of inheritance of virulence in G. rostochiensis against the resistance H_1 gene of S. tuberosum ssp. andigena CPC 1673 were carried out with the avirulent line Ro_1-19 and the virulent line Ro_5-22 (Janssen et al., 1990b), on the susceptible cultivar 'Eigenheimer' and the resistant cultivar 'Saturna'. Experiments were carried out in Petri dishes and pots, in a controlled environment room at 18 °C and 16 h light.

Reciprocal crosses (Ro_5-22 $\frac{x}{x}$ Ro_1-19) were made by placing a male on the gelatinous matrix of a female. This was done each time on separate Petri dishes to ensure that the female was not fertilized by more than one male. Males of both lines were reared on cultivar 'Eigenheimer' in sandy loam in clay pots (700 ml), inoculated with 50 cysts, and harvested after about 30 days with an elutriator (Oostenbrink, 1960). Virgin females were obtained from potato roots grown on water agar (Mugniery & Person, 1976) inoculated with one larva per root tip per Petri dish. Females of Ro_1-19 and Ro_5-22 were reared on roots of the cultivars 'Eigenheimer' and 'Saturna' respectively.

The virulence of the larvae from 30 F_1 cysts of both crosses were tested in Petri dishes by inoculating 200 larvae (two per root tip), unless stated otherwise (Mugniery & Person, 1976; Janssen et al., 1990a). Virulence is expressed as a percentage of the number of females that developed on the resistant cultivar relative to the number of females on the susceptible cultivar.

Larvae of another 30 F_1 cysts were selfed by inoculating cultivar 'Eigenheimer' with larvae of one cyst per clay pot. Larvae were artificially hatched by cutting cysts in half before inoculation (Janssen et al., 1987). Newly formed cysts were recovered from the wet soil with an elutriator (Kort, 1960), using a upstream of 4.5 l min^{-1}, and collected on a 0.30 x 3.65 mm pore sieve. Nine F_2 lines per cross with more than 50 newly formed cysts were selected for virulence tests. Larvae were obtained by artificial hatching.

Results

The virulence percentages of the parent lines Ro_1-19 and Ro_5-22 and their reciprocal crosses are shown in Table 1. Ro_1-19 x Ro_5-22 is fully avirulent for the H_1 resistance gene. Ro_5-22 x Ro_1-19 has a virulence level of 0.2%; out of the 600 inoculated larvae on the resistant cultivar 'Saturna' one cyst developed.

Table 1. Virulence percentages of virulent phenotypes for the avirulent line Ro_1-19, the virulent line Ro_5-22 and the F_1 of their crosses on cultivar 'Saturna'

Parents and F_1	Virulence percentages	
P Ro_1-19	0	
F_1 Ro_1-19 ♀ x Ro_5-22 ♂	0	a
F_1 Ro_5-22 ♀ x Ro_1-19 ♂	0.2	a
P Ro_5-22	100.2	

a) 600 larvae (two larvae per root tip) on cultivar 'Saturna'

The numbers of newly formed cysts per clay pot after inoculation with one F_1 cyst are shown in table 2. The multiplication rates varied from 1 to 251. Thirty six percent of the F_1 cysts produced more than 50 new cysts and only 8% less than 10. Line S8 produced 911 new cysts, an unexpected and extremely large number which may have resulted from the development of a second generation in this pot due to crushed cysts after an inspection of the root ball. This line was omitted from the experiment.

Eighteen of the F_2 lines with 50 cysts or more were considered for a virulence test but only eight lines of the cross Ro_1-19 x Ro_5-22 (E numbers) and seven lines of the cross Ro_5-22 x Ro_1-19 (S numbers) produced more than 400 larvae, the minimum number needed for the virulence test (Table 3). One F_2 line did not hatch at all and two other lines gave only 27 and 158 larvae.

Table 2. Numbers of newly formed cysts (F_2) after inoculation of one F_1 cyst per clay pot on cultivar 'Eigenheimer'. E lines: cross Ro_1-19 ♀ x Ro_5-22 ♂; S lines: cross Ro_5-22 ♀ x Ro_1-19 ♂

Line	Cysts	Line	Cysts	Line	Cysts	Line	Cysts	Line	Cysts	Line	Cysts
E1	22	E11	27	E21	40	S1	38	S11	102	S21	50
E2	74	E12	10	E22	218	S2	100	S12	48	S22	45
E3	8	E13	26	E23	114	S3	59	S13	33	S23	13
E4	1	E14	175	E24	2	S4	49	S14	18	S24	14
E5	25	E15	7	E25	17	S5	30	S15	28	S25	50
E6	62	E16	39	E26	43	S6	156	S16	57	S26	46
E7	59	E17	97	E27	64	S7	114	S17	10	S27	19
E8	45	E18	79	E28	30	S8	(911)[a]	S18	36	S28	57
E9	45	E19	38	E29	36	S9	199	S19	34	S29	1
E10	62	E20	51	E30	15	S10	251	S20	28	S30	135
Sum					1515						1816
$\bar{x}$					51.0						62.8
σ					48.4						58.8

a) not included

On the basis of the number of cysts developing on cultivar 'Eigenheimer' relative to that number on cultivar 'Saturna', frequencies of virulent genotypes were calculated (Table 3). These frequencies in both crosses did not differ significantly (at the 95% confidence level) from the expected virulence frequency of 25% belonging to a 3:1 segregation for avirulence (AA and Aa) and virulence (aa).

Assuming a monogenic basis of virulence, the relative frequencies of avirulent (AA and Aa) and virulent (aa) genotypes in the F_2's are 0.75 and 0.25, respectively. The ratio of the numbers of cysts developing on cultivar 'Eigenheimer' versus the number developing on cultivar 'Saturna' equals then 4 (AA + Aa + aa) : 1 (aa). Table 4 presents the Chi-square test. None of the individual lines showed significant deviations from the expected frequencies. Testing for heterogeneity

between lines within crosses, and between crosses revealed no heterogeneity between lines within either cross, but a significant difference was found comparing the pooled E and S lines. This difference resulted from constantly higher numbers of observed virulent genotypes in the S lines than expected on basis of the hypothesis. Though the deviation of the pooled S lines is significant, it is small as is confirmed by the absence of overall heterogeneity between lines.

Table 3. Numbers of cysts of the F_2 lines in the virulence tests formed on cultivar 'Saturna' and cultivar 'Eigenheimer', and the calculated virulence frequencies. E lines: cross Ro_1-19 ♀ x Ro_5-22 ♂; S lines: cross Ro_5-22 ♀ x Ro_1-19 ♂

Line no	Numbers of cysts formed on		Virulence frequencies
	Eigenheimer	Saturna	
E2	110	25	22.7
E6	123	31	25.2
E14	143	33	23.1
E17	147	36	24.5
E18	140	32	22.9
E22	141	28	19.9
E23	138	33	23.9
E27	121	34	28.1
S2	116	37	31.9
S6	135	36	26.7
S7	136	40	29.4
S9	120	37	30.8
S10	143	41	28.7
S11	151	42	27.8
S30	138	41	29.7
$\bar{x}$			26.4
σ			3.5

Table 4. Segregation for virulence in the F_2 generation of the reciprocal crosses of an avirulent (Ro_1-19) and a virulent (Ro_5-22) line for cultivar 'Saturna' and the heterogeneity test; E lines: Ro_1-19 ♀ x Ro_5-22 ♂; S lines: Ro_5-22 ♀ x Ro_1-19 ♂

	Line no	avirulent		virulent		X_1^2 for 4:1	P-value
		observed	expected	observed	expected		
	E2	110	108	25	27	0.1852	0.50–0.70
	E6	123	123.2	31	30.8	0.0016	0.95–1.00
	E14	143	140.8	33	35.2	0.1719	0.50–0.70
	E17	147	146.4	36	36.6	0.0123	0.90–0.95
	E18	140	137.6	32	34.4	0.2093	0.50–0.70
	E22	141	135.2	28	33.8	1.2441	0.20–0.30
	E23	138	136.8	33	34.2	0.0526	0.80–0.90
	E27	121	124	34	31	0.3629	0.50–0.70
Total E		1063	1052	252	263	0.5751	0.30–0.50
	S2	116	122.4	37	30.6	1.6732	0.10–0.20
	S6	135	136.8	36	34.2	0.1184	0.70–0.80
	S7	136	140.8	40	35.2	0.8182	0.30–0.50
	S9	120	125.6	37	31.4	1.2484	0.20–0.30
	S10	143	147.2	41	36.8	0.5992	0.30–0.50
	S11	151	154.4	42	38.6	0.3744	0.50–0.70
	S30	138	143.2	41	35.8	0.9441	0.30–0.50
Total S		939	970.4	274	242.6	5.0802	0.01–0.05
Total E+S		2002	2022.4	526	505.6	1.0289	0.30–0.50

Heterogeneity test:	Source	X^2	d.f.	P-value
	Between E-lines	1.6648	7	0.95–1.00
	Between S-lines	0.6957	6	0.95–1.00
	E versus S	4.6264	1	0.01–0.05
	Total	6.9869	14	0.90–0.95

Discussion

Gene-for-gene relationships have been suggested for a wide range of pathogens, including bacteria, fungi, viruses, insects, nematodes and plant parasitic plants. However, the number of host-parasite systems for which Mendelian segregation patterns of both interacting partners have been analyzed, is relatively small (Crute, 1985). A formal proof accordings to Flor's criterion (Sidhu, 1975) has been obtained for associations of only 15 fungi/plants and one insect/plant. In this study we analyzed the segregation patterns of virulence in potato cyst nematodes for the H_1 gene and showed that the gene-for-gene concept applies to one of the potato cyst nematodes and its host. The avirulence of the F_1 and the 3:1 segregation in the F_2 demonstrates that virulence is inherited at a single locus and is recessive to avirulence. As might be expected from the epigenic nature of the sex determination in potato cyst nematodes (Trudgill, 1967; Mugniery, 1982, 1985; Mugniery & Fayet, 1981, 1984), the reciprocal crosses revealed no sex-linked inheritance.

The virulence percentage in the F_1 of the Ro_5-22 (aa) x Ro_1-19 (AA) cross showed a 0.2% deviation from the expected complete avirulence (Table 1). This observation does not invalidate the gene-for-gene hypothesis. A plausible explanation for such a low virulence level may be, as discussed earlier (Janssen et al., 1990b), that the resistance mechanism conferred by the H_1 gene is not absolute. Arguments for heterozygosity in the Ro_1-19 line lack a numerical basis. If one out of the 30 F_1 cysts in the virulence test had been the result of a Ro_5-22 (aa) x Ro_1-19 (Aa) cross, the virulence percentage should have been 1.7. The absence of cysts in the F_1 of the reciprocal cross further supports the argument of escapers. Another explanation for this deviation may be found in some kind of maternal effect operating in the Ro_5-22 line, which also could explain the small but significant difference from the 4:1 segregation of the pooled S lines in the F_2 (Table 4).

Person (1959) stated that genetic data for both interacting partners are not required in evaluating a possible gene-for-gene mechanism. His concept (Sidhu, 1975) is based on the pattern of interactions

generated when several host varieties are inoculated with different cultures of the pathogen. The concept presupposes two alternative conditions of expression in the host (resistance versus susceptibility) and the parasite (virulence versus avirulence). It is tempting to extrapolate this concept to the various sources of resistance against plant parasitic nematodes. However, in plant parasitic nematodes with an amphimictic mode of reproduction isolates which are fixed for virulence alleles are hardly available. Interaction patterns of e.g. potato cyst nematodes and their host often result in an array of multiplication rates ranging from 0 to more than 60 (e.g. Kort et al., 1977) thus masking possible gene-for-gene relationships. However, regardless of these specific problems in amphimictic nematode species, Person's approach is less precise than Flor's criterion. In this report we have chosen the Mendelian approach to obtain a solid basis for tracing a gene for (a)virulence in potato cyst nematodes.

References

Crute, R. (1985). The genetic base of relationships between microbial parasites and their hosts. In: Fraser, R.S.S. (Ed.). Mechanisms of resistance to plant diseases, Martinus Nijhoff/Dr W. Junk Publishers, Dordrecht: 80-142.

Dunnett, J.M. (1961). Inheritance of resistance to potato-root eelworm in breeding lines stemming from Solanum multidissectum Hawkes. Rep. Scottish Breed. Stn.,: 39-46.

Ellenby, C. (1952). Resistance to the potato root eelworm, Heterodera rostochiensis Wollenweber. Nature, 170: 1016.

Flor, H.H. (1956). The complementary genetic systems in flax and flax rust. Adv. Genet., 8: 29-54.

Goffart, H. & Ross, H. (1954). Untersuchungen zur Frage der Resistenz von Wildarten der Kartoffel gegen den Kartoffel nematoden (Heterodera rostochiensis Wr.). Zuchter, 24: 193-201.

Howard, H.W. (1959). Biotypes of potato root eelworm in Great Britain. Tagber. Dt. Akad. Landw-Wiss. Berl., 20: 95-106.

Howard, H.W., Cole C.S. & Fuller J.M. (1970). Further sources of resistance to Heterodera rostochiensis Woll. in the Andigena potato. Euphytica, 19: 210-216.

Huijsman, C.A. (1955). Breeding for resistance to the potato root eelworm II. Euphytica, 4: 133-140.

Huijsman, C.A. (1960). Some data on the resistance against the potato root-eelworm (Heterodera rostochiensis W.) in Solanum kurtzianum. Euphytica, 9: 185-190.

Huijsman, C.A. (1974). Host-plants for Heterodera rostochiensis Woll. and the breeding for resistance. EPPO Bull., 4: 501-509.

Huijsman, C.A. & Lamberts, H. (1972). Breeding for resistance to the potato cyst-nematode in the Netherlands. In: French, E.R. (Ed.). Prospects for the Potato in the Developing World, Int. Potato Center, Lima, Peru: 161-171.

Janssen, R., Bakker, J. & Gommers, F.J. (1987). Circumventing the diapause of potato cyst nematodes. Neth. J. Pl. Path., 93: 107-113.

Janssen, R., Bakker, J. & Gommers, F.J. (1990 a). Assessing intra-specific variations in virulence in Globodera rostochiensis and G. pallida. Revue Nématol., 13: in press.

Janssen, R., Bakker, J. & Gommers, F.J. (1990 b). Selection of lines of Globodera rostochiensis virulent and avirulent to the H_1 resistance gene in Solanum tuberosum ssp. andigena CPC 1673. Revue Nématol., 13: in press.

Jones, F.G.W. & Parrott, D.M. (1965). The genetic relationships of pathotypes of Heterodera rostochiensis Woll. which reproduce on hybrid potatoes with genes for resistance. Ann. appl. Biol., 56: 27-36.

Jones, F.G.W., Parrott, D.M. & Perry, J.N. (1981). The gene-for-gene relationship and its significance for potato cyst nematodes and their solanaceous hosts. In: Zuckerman, B.M. & Rohde, R.A. (Eds.). Plant Parasitic Nematodes, Vol. III, Academic Press, Inc., New York: 23-36.

Jones, F.G.W., Parrott, D.M. & Ross, G.S.J. (1967). The population genetics of the potato cyst-nematode, Heterodera rostochiensis: mathematical models to simulate the effects of growing eelworm-resistant potatoes bred from Solanum tuberosum ssp. andigena. Ann. appl. Biol., 60: 151-171.

Kort, J. (1960). A technique for the extraction of Heterodera cysts from wet soil and for the estimation of their eggs and larval content. Versl. Meded. plziektenk. Dienst, Wageningen, 233: 3-7.

Kort, J., Jaspers, C.P. & Dijkstra, D.L. (1972). Testing for resistance to pathotype C of Heterodera rostochiensis and the practical application of Solanum vernei hybrids in the Netherlands. Ann. app. Biol., 71: 289-294.

Kort, J., Ross, H., Rumpenhorst, H.J. & Stone, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst-nematodes Globodera rostochiensis and G. pallida. Nematologica, 23: 333-339.

Mugniery, D. (1982b). Influence de l'hôte sur le developpement et l'expression du sexe chez un nématode phytoparasite à déterminisme sexuel épigénique, Globodera pallida Stone. C. r. hebd. Séanc Acad. Sci., Paris, 294, Sér. III: 49-52.

Mugniery, D. (1985). Résistance des solanacées à Globodera et influence de certains facteurs sur les résultats des tests de résistance. EPPO Bull., 15: 199-206.

Mugniery, D. & Fayet, G. (1981). Détermination du sexe chez Globodera pallida Stone. Revue Nématol., 4: 41-45.

Mugniery, D. & Fayet, G. (1984). Détermination du sexe de Globodera rostochiensis Woll. et influence des niveaux d'infestation sur la pénétration, le développement et le sexe de ce nématode. Revue Nématol., 7: 233-238.

Mugniery, D. & Person, F. (1976). Méthode d'elevage de quelques nématodes à kystes du genre Heterodera. Sci. Agron., Rennes, 217-220.

Oostenbrink, M. 1960. Estimating nematode populations by some selected methods. In: Sasser, J.N. & W.R. Jenkins, W.R. (Eds.). Nematology, Chapel Hill, Univ. of North Carolina Press: 85-102.

Parrott, D.M. (1981). Evidence for gene-to-gene relationships between resistance gene H_1 from Solanum tuberosum ssp. andigena and a gene in Globodera rostochiensis, and between H_2 from S. multidissectum and a gene in G. pallida. Nematologica, 27: 372-384.

Parrott, D.M. & Berry, M.M. (1974). Inheritance of ability to overcome resistance. Rothamsted Exp. Stn. Rep. 1973, Part 1: 152-153.

Person, C. (1959). Gene-for-gene relationship in host:parasite systems. Can. J. Bot., 37: 1101-1130.

Ross, H. (1962). Über die Vererbung der Resistenz gegen den Kartoffel Nematoden (Heterodera rostochiensis Woll.) in Kreuzungen von Solanum famatina Bitt. et Wittm. mit Solanum tuberosum L. und S. chacoense Bitt. Zuchter, 32: 74-80.

Ross, H. (1969). Zuchtung von Kartoffelsorten mit Resistenz gegen Heterodera rostochiensis Woll. Mitt. Biol. Bundesanst. Land. Forstwirtsch. Berlin-Dahlem, 136: 59-64.

Ross, H. (1972). Improvement of the potato in Germany for resistance to the two species of cyst nematode. In: French, E.R. (Ed.). Prospects for the Potato in the Developing World, Int. Potato Center, Lima, Peru: 181-190.

Sidhu, G.S. (1975). Gene-for-gene relationships in plant parasitic systems. Sci. Prog. Oxf., 62: 467-485.

Toxopeus, H.J. & Huijsman, C.A. (1952). Genotypical background of resistance to Heterodera rostochiensis in Solanum tuberosum var. andigenum. Nature, 170: 1016.

Toxopeus, H.J. & Huijsman, C.A. (1953). Breeding for resistance to potato root eelworm I. Euphytica, 2: 180-186.

Trudgill, D.L. (1967). The effect of environment on sex determination in Heterodera rostochiensis. Nematologica, 13: 263-272.

CHAPTER V

THE EFFECT OF HETEROZYGOSITY FOR (A)VIRULENCE GENES ON THE DEVELOPMENT OF POTATO CYST NEMATODES

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Abstract

The genetic constitution of avirulent larvae (Aa and AA) of Globodera rostochiensis has no substantial influence on the development into adults on Solanum tuberosum ssp. andigena CPC 1673 containing the H_1 resistance gene. None of the AA larvae and only 0.3% of the Aa larvae were able to develop into females. The H_1 gene was only moderately effective against males. Both AA and Aa larvae were able to develop into males. The heterozygous males (Aa) had no real advantage over homozygous males (AA).

Introduction

A crucial process in the development of potato cyst nematodes is the induction and maintenance of a syncytium, which regulates the transfer of nutrients towards the nematode (e.g. Gommers, 1981; Zacheo, 1986). After the induction of a feeding site the second stage larva becomes sedentary and progresses through third and fourth developmental stages to adult. Sex differentation, which becomes visible in the third developmental stage, is epigenic and is mainly influenced by

nutritional factors (Janssen, et al., 1987; Trudgill, 1967; Mugniery, 1982, 1985; Mugniery & Fayet, 1981, 1984). The imbalance of the adult sex ratio towards males in resistant plants (e.g. Turner & Stone, 1984) is thought to result from the poor nutritional potential of the syncytia. In incompatible combinations the syncytia remain small and become surrounded by necrotic tissue, which limits the transport of nutrients from plant to nematode (Kühn, 1958; Huijsman et al., 1969; Rice et al., 1985). In contrast with males, females cannot develop because of their greater nutrional needs.

Mendelian segregation patterns followed by the development of females on plants having the H_1 resistance gene from Solanum tuberosum ssp. andigenum CPC 1673, showed that virulence in Globodera rostochiensis is inherited at a single locus and is recessive to avirulence (Janssen et al., 1990c). The resistance mechanism conferred by the H_1 gene is not absolute and double dominant avirulent larvae (AA) incidentally develop into females. The underlying process is unclear and it is also unknown wether heterozygous larvae (Aa) have an advantage over double dominant larvae (AA) to escape from the resistance mechanism. Also the fate of the double dominant (AA) and the heterozygous larvae (Aa) has never been subjected to detailed analyses. It was hypothesized that both genotypes are able to develop into males on the resistant plant and that the heterozygous genotype has no selective advantage over the double dominant genotype (Jones et al., 1967).

In this study we analysed the effects of the genetic constitution of avirulent genotypes (Aa and AA) on the development of larvae into adults on plants having the H_1 resistance gene.

Material and methods

Air-dried cysts of the avirulent line Ro_1-19 and the virulent line Ro_5-22 (Janssen et al., 1990b) were pre-soaked in tap water and after one week the larvae were hatched with root diffusate of the cultivar Bintje. Newly formed fresh F_1 cysts were artificially hatched (Janssen et al., 1987).

Roots of sprouts of tuber segments of the susceptible cultivar

'Eigenheimer' and cultivar 'Saturna' carrying the H_1 gene from S.tuberosum ssp. andigena CPC 1673 were grown on water agar in Petri dishes (Mugniery & Person, 1976). The Petri dishes were kept in the dark at 18°C. Freshly hatched larvae or larvae stored up to three days at 4°C, were used. Two larvae were inoculated per root tip (Janssen et al., 1990a). The inoculum size was 400 larvae (200 root tips) per treatment.

The F_1 of line Ro_5-22 and line Ro_1-19, (Ro_5-22xRo_1-19), was obtained by placing one male on the gelatinous matrix of the female. Males from Ro_1-19 were reared on cultivar 'Eigenheimer' in clay pots (700 ml) in sandy loam soil inoculated with 50 cysts in a controlled environment with 16h light and at 18 °C. A slow release N-P-K granulate fertilizer (Osmocote ^{R}) was added. Males were harvested after about 30 days with an elutriator (Oostenbrink, 1960). Females were reared on roots of cultivar 'Saturna' grown on water agar (Janssen et al.,1990a; Mugniery & Person, 1976) by inoculating one larvae per root tip per Petri dish.

Ten days after inoculation, root systems and adhering agar gel were submerged in beaker glasses filled with tap water, by piercing a rod through the tuber segment and the agar gel. Contact between the segment and water was avoided. After twenty days, the tuber segment with the root system and adhering agar was returned to the Petri dish to assess accurately the numbers of females. Males were counted from the beaker glasses.

Results

The number of larvae developing into adults on cultivar 'Eigenheimer' ranged from 228 (Ro_1-19), 266 (Ro_5-22) to 321 (F_1), indicating that the two lines and the F_1 differ in their vitality. The effect of the hosts in compatible combinations on the growth of adults was less distinct. The numbers of adults produced by Ro_5-22 was 266 on cultivar 'Eigenheimer' and 282 on cultivar 'Saturna'.

In compatible combinations the male-female ratio averaged 5.5%, whereas this figure was 143% in the two incompatible combinations. None of the homozygous avirulent larvae (AA) and only one heterozygous avirulent larvae (Aa) was able to develop into a female on cultivar

'Saturna'. The differences between the two genotypes were more pronounced considering the development into males. On cultivar 'Saturna' the number of heterozygous males (94) was nearly twice as high as the number of double dominant males (49). These differences are also significant mathematically when the numbers of males are expressed as a percentage of the total number of adults that developed on 'Eigenheimer' (see Table 1).

Table 1. Number of adults of Ro_1-19 (AA), Ro_5-22 ♀ x Ro_1-19 ♂ (Aa) and Ro_5-22 (aa) that developed on susceptible cultivar 'Eigenheimer' and resistant cultivar 'Saturna' after inoculating 400 larvae per combination

pathotype (genotype)	cultivar	♀♀	♂♂	total adults	% ♂♂ over total adults on Eigenheimer
Ro_1-19 (AA)	Eigenheimer	220	8	228	
	Saturna	0	49		21.5 a
Ro_5-22xRo_1-19 (Aa)	Eigenheimer	307	14	321	
	Saturna	1	94		29.3 b
Ro_5-22 (aa)	Eigenheimer	260	6	266	
	Saturna	253	29		10.9 c

Figures with different letter are significant at $P<0.005$

Discussion

Various studies have shown that males of avirulent populations can develop on plants having the H_1 resistance gene (e.g. Jones, 1954; Ouden, 1958; Turner & Stone, 1984; Trudgill et al, 1967; Forrest et al., 1986). However, in these studies the AA and Aa genotypes were not tested separately. We demonstrated that both AA and Aa genotypes are able to develop into males. It is also apparent that the resistance conferred by the H_1 gene is to a certain extent also operating on the development of males. The total number of adults in compatible combinations, predominantly females, is much higher than the number of adults, predominantly males, in incompatible combinations. For example, the number of adults of line Ro_1-19 on cultivar 'Eigenheimer' is 228, whereas this figure is 49 on cultivar 'Saturna'. These data demonstrate that larvae able to develop into females on cultivar 'Eigenheimer' do not necessarily develop into males on cultivar 'Saturna'.

As already discussed in a previous report (Janssen et al., 1990b), the resistance of the H_1 gene is probably not absolute. Our data indicate that heterozygous larvae have no or only a slight advantage over homozygous dominant larvae to develop into females on plants carrying the H_1 resistance gene. Only one Aa larva out of the 400 larvae inoculated developed into a female and none of the avirulent AA larvae.

The number of males of the heterozygous F_1 (94) on cultivar 'Saturna' is much higher than the number of homozygous avirulent males of line Ro_1-19 (49). However, the absolute numbers seem not suitable for a proper evaluation of a possible selective advantage of the heterozygous larvae (Aa) over homozygous dominant larvae (AA) to develop into males. The F_1 larvae are more vital than the larvae of Ro_1-19. These differences in vitality may also explain the larger numbers of males of the F_1 on cultivar 'Saturna'. The increased vitality of the F_1 larvae may have resulted from heterosis or from the fact that the larvae of the F_1 were obtained by artificial hatching from young cysts, whereas the larvae of the two inbred lines were hatched from one year old air-dried cysts. The relative number of

males, calculated as a percentage of the number of adult females and males on cultivar 'Eigenheimer', seems a more appropriate measure, because the contribution of vitality is minimized in these figures. As shown by the relative numbers in Table 1 the F_1 larvae (29,3%) may have a slight advantage over the larvae of Ro_1-19 (21.5%) to develop into males. However, it can not be excluded that differences in vitality of the larvae, also interfer with these relative numbers. In any case, it seems feasible to conclude, that if heterozygous larvae have a selective advantage, the selection pressure is small and has no large consequences for the population genetics of virulence. Simulation models, which assert from the assumption that AA and Aa genotypes have an equal chance to develop into males on plants having the H_1 gene, are sufficiently accurate to predict the behaviour of virulence in field populations (Jones & Perry, 1978; Jones et al, 1981; Spitters & Ward, 1988).

Biochemical explanations for a gene-for-gene system are often based either on the specificity of the incompatible combination or compatible combination. In the first model, the elicitor-receptor theory, the avirulence allele produces an elicitor which triggers the hypersensitive reaction (Keen, 1981). In the alternative model of induced susceptibility, the virulence allele produces a substance, which blocks the biosynthetic pathway of the hypersensitive response (Ouchi et al., 1976). Both models are also applicable to the development of females and males on plants having the H_1 resistance gene. Our data indicate that heterozygosity does not result in a less outspoken or delayed hypersensitive reaction. The presence of one avirulent allele in the heterozygous larvae resulting either in a elicitor (first model) or an incomplete blocking mechanism (second model), leads to a host reaction which is comparable with the reaction evoked by the double dominant larvae.

Acknowledgements

We thank Tjaart W. Hofman and Harm Schipper for carrying out the preliminary experiments.

References

Forrest, J.M.S., Trudgill, D.L. & Cotes, L.M. (1986). The fate of juveniles of Globodera rostochiensis and G. pallida in roots of susceptible and resistant potato cultivars with gene H_1. Nematologica, 32: 106-114.

Gommers, F.J. (1981). Biochemical interactions between nematodes and plants and their relevance to control. Helminth. Abstr., Series B, 50: 9-24.

Huijsman, C.A., Klinkenberg, C.H. & Ouden, den H. (1969). Tolerance to Heterodera rostochiensis Woll. among potato varieties and its relation to certain characteristics of root anatomy. Eur. Potato J., 12: 134-147.

Janssen, R., Bakker, J. & Gommers, F.J. (1987). Circumventing the diapause of potato cyst nematodes. Neth. J. Pl. Path., 93: 107-113.

Janssen, R., Bakker, J. & Gommers, F.J. (1990 a). Assessing intraspecific variations in virulence in Globodera rostochiensis and G. pallida. Revue Nématol., 13: in press

Janssen, R., Bakker, J. & Gommers, F.J. (1990 b). The selection of lines of Globodera rostochiensis virulent and avirulent to the H_1 resistance gene in Solanum tuberosum ssp. andigena CPC 1673. Revue Nemátol., 13: in press.

Janssen, R., Bakker, J. & Gommers, F.J. (1990 c). Mendelian proof for a gene-for-gene relationship between virulence of Globodera rostochiensis and the H_1 resistance gene in Solanum tuberosum ssp. andigena CPC 1673. Revue Nemátol., 13: accepted.

Jones, F.G.W. (1954). First steps in breeding for resistance to potato root eelworm. Ann. appl. Biol., 41: 348-353.

Jones, F.G.W. & Perry, J.N. (1978). Modelling populations of cyst-nematodes (Nematoda: Heteroderae). J. appl. Ecol., 15: 349-371.

Jones, F.G.W., Parrott, D.M. & Perry, J.N. (1981). The gene-for-gene relationship and its significance for potato cyst nematodes and their solanaceous hosts. In: Zuckerman, B.M. & Rohde, R.A. (Eds.). Plant Parasitic Nematodes, Vol. III, Academic Press, Inc., New York: 23-36.

Jones, F.G.W., Parrott, D.M. & Ross, J.S. (1967). The population genetics of potato cyst nematode, Heterodera rostochiensis: mathematical models to simulate the effects of growing eelworm-resistant potatoes bred from Solanum tuberosum ssp. andigena. Ann. appl. Biol., 60: 151-171.

Keen, N.T. (1981). Specific recognition in gene-for-gene host-parasite systems. In: Williams, P.H. & Ingram, D. (Eds.). Advances in Plant Pathology, Vol. I, Academic Press, New York: 35-82.

Kühn, H. (1958). Über die Abwehrnekrose eines Kartoffelbastardes gegen den Kartoffelnematoden (Heterodera rostochiensis Wr. in Solanum tuberosum subsp. andigena [Juz. et Buk.] HwK. x Solanum tuberosum L.). Z. Pfl. Krankh. 65: 465-472.

Mugniery, D. (1982). Physiology des invertèbrès. Influence de l'hôte sur le développment et l'expression du sexe chez un nématode phytoparasite à détermine sexuel épigénique, Globodera pallida Stone. C. R. Hebd. Séanc. Acad. Sci., Paris, 294, Sér. III: 49-52.

Mugniery, D. (1985). Résistance des solanacées à Globodera et influence de certains facteurs sur les résultats des tests de résistance. EPPO Bull., 15: 199-206.

Mugniery, D. & Fayet, G. (1981). Détermination du sexe chez Globodera pallida Stone. Revue Nématol., 4: 41-45.

Mugniery, D. & Fayet, G. (1984). Détermination du sexe de Globodera rostochiensis Woll. et influence des niveaux d'infestation sur la pénétration, le développement et le sexe de ce nématode. Revue Nématol., 7: 233-238.

Mugniery, D. & Person, F. (1976). Méthode d'élevage de quelques nématodes a kystes du genre Heterodera. Sci. Agron., Rennes, 217-220.

Oostenbrink, M. (1960). Estimating nematode populations by some selected methods. In: Sasser, J.N. & Jenkins, W.R. (Eds.). Nematology, Chapel Hill, Univ. of North Carolina Press: 85-102

Ouchi, S., Hibino, C. & Oku, H. (1976). Effect of earlier inoculation on the establishment of a subsequent fungus as demonstrated in powdery mildew of barley by a triple inoculation procedure. Physiol. Plant Pathol., 9: 25-32.

Ouden, H. den. (1958). Onderzoek over Heterodera rostochiensis. IPO-Jaarverslag, 120.

Rice, S.L., Leadbeater, B.S.C. & Stone, A.R. (1985). Changes in cell structure in roots of resistant potatoes parasitized by potato cyst-nematodes. I. Potatoes with resistance gene H_1 derived from Solanum tuberosum ssp. andigena. Physiol. Plant Pathol., 27: 219-234.

Spitters, C.J.T. & Ward, S.A. (1988). Evaluation of breeding strategies for resistance to potato cyst nematodes using a population dynamic model. Euphytica, suppl.: 87-98.

Trudgill, D.L. (1967). The effect of environment on sex determination in Heterodera rostochiensis. Nematologica, 13: 263-272.

Trudgill, D.L., Webster, J.M. & Parrott, D.M. (1967). The effect of resistant solanaceous plants on the sex ratio of Heterodera rostochiensis and the use of the sex ratio to assess the frequency and genetic constitution of pathotypes. Ann. appl. Biol., 60: 421-428.

Turner, S.J. & Stone, A.R. (1984). Development of potato cyst-nematodes in roots of resistant Solanum tuberosum ssp. andigena and S. vernei hybrids. Nematologica, 30: 324-332.

Zacheo, G., (1986). Biochemical reactions and interactions. In: Lamberti, F. & Taylor, C.E. (Eds.). Cyst Nematodes, Plenum Press, New York: 163-177.

SAMENVATTING EN PERSPECTIEVEN

Het centrale thema van het onderzoek was de bestudering van de genetische achtergronden van virulentie en avirulentie van Globodera rostochiensis voor het H_1 resistentiegen in Solanum tuberosum ssp. andigena CPC 1673. Deze monogene resistentie is op dit moment de belangrijkste vorm van resistentie in consumptie- en fabrieks-aardappelrassen in West-Europa. Net als bij andere planteparasitaire nematoden werd genetisch onderzoek aan aardappelcysteaaltjes belemmerd door problemen bij het uitvoeren van kruisingsexperimenten. Het genetisch onderzoek dat desondanks werd uitgevoerd kenmerkte zich door tijdrovende experimenten vanwege de diapauze van bijna één jaar, niet goed te controleren massa kruisingen, en het gemis aan populaties met nauwkeurig gedefinieerde niveau's van virulentie. Genetische modellen konden slechts getoetst worden aan de hand van het gedrag van veld-populaties. Het model van Jones, gebaseerd op het gen-om-gen systeem dat voor het eerst gevonden werd bij de plant/schimmel interactie vlas/roest, beschrijft op een redelijke wijze de virulentie-ontwikkeling van veldpopulaties. In zijn theorie zijn virulente individuen voor het H_1 resistentiegen homozygoot recessief.

In de eerste drie hoofdstukken van dit proefschrift zijn de methoden beschreven waarmee binnen een acceptabele tijd genetische experimenten uitgevoerd kunnen worden. Genetische analyses van virulentie in aardappelcysteaaltjes vormen het onderwerp van de laatste twee hoofd-stukken.

In het eerste hoofdstuk wordt een methode beschreven waarmede achter-eenvolgende generaties aardappelcysteaaltjes gekweekt kunnen worden door de diapauze te omzeilen. Hiertoe worden de cysten gekweekt op wortels van aardappelspruiten in Petrischalen met wateragar of in grond en zorgvuldig vochtig gehouden. De larven worden uit de cyst gelokt door de cysten met een scalpel te halveren of voorzichtig door te drukken zonder de eieren te beschadigen ("crushen") en deze vervolgens te incuberen in lokstof. Op deze wijze wordt ongeveer 40% van de cysteïnhoud gelokt. Deze behandeling heeft geen nadelige invloeden op de vitaliteit van de larven en de hieruit ontwikkelde

mannetjes en vrouwtjes. Dit geldt zowel voor eieren uit cysten gekweekt in Petrischalen als in grond. Op deze wijze is het mogelijk drie tot vijf generaties per jaar in potten te kweken en vijf tot zes generaties in Petrischalen.

Een essentieel onderdeel voor het uitvoeren van het genetisch onderzoek was een gestandaardiseerde en nauwkeurige bepaling van de virulentie in populaties (hoofdstuk II). Hiervoor werden cysten in Petrischalen op wortels van aardappelspruiten gekweekt. Optimale resultaten werden verkregen door twee larven per wortelpunt te inoculeren. Het percentage virulente individuen in diverse populaties werd bepaald door larven op een vatbare en een resistente cultivar te inoculeren en het aantal gevormde vrouwtjes op de resistente cultivar uit te drukken in percentages van het aantal gevormde vrouwtjes op de vatbare cultivar. Deze percentages varieerden aanzienlijk in populaties van pathotypen van zowel G. rostochiensis als G. pallida. Ter bepaling van de nauwkeurigheid van de methode werd een vergelijking gemaakt met de gangbare pathotypetoets op basis van P_f/P_i bepalingen. De waarden voor de variatiecoëfficient geven duidelijk aan dat de methode in Petrischalen beter geschikt is voor het bepalen van virulentieniveau's dan aan de hand van potexperimenten. Voor verder onderzoek werden de populaties Ro_1-Mierenbos en Ro_5-Harmerz uitgekozen met respectievelijk <0,1% en 84.6% virulente individuen voor het H_1 resistentiegen.

Het kweken van cysten onder geconditioneerde omstandigheden en de hierboven beschreven techniek maakten het mogelijk om na gecontroleerde enkelvoudige kruisingen virulente en avirulente lijnen voor het H_1 resistentiegen te selecteren (hoofdstuk III). Voor deze kruisingen werden mannetjes in grond en vrouwtjes op wortels in Petrischalen gekweekt. Kruisingen werden uitgevoerd door één mannetje te plaatsen op de gelatineuze matrix van het vrouwtje. In een groot aantal F_2 en F_3 lijnen werden de virulentieniveau's bepaald. Uit populatie Ro_1-Mierenbos en Ro_5-Harmerz werden respectievelijk een avirulente en een virulente lijn geselecteerd, die gebruikt werden voor een nadere genetische analyse.

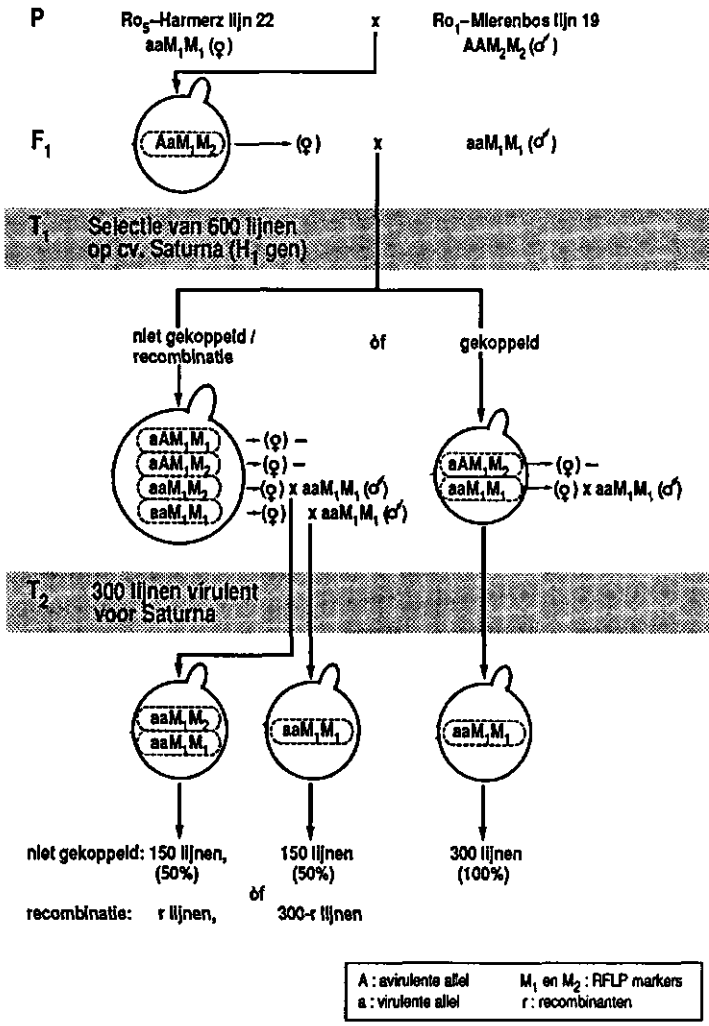
Reciproke kruisingen tussen individuen van een avirulente en een virulente lijn gaven avirulente nakomelingen (F_1). Zelfbevruchting van

de F_1 leverde in de F_2 25% virulente en 75% avirulente individuen. Deze 1:3 uitsplitsing betekent dat virulentie recessief (a) is; virulente individuen zijn homozygoot recessief (aa), en avirulente individuen homozygoot dominant (AA) of heterozygoot (Aa). Bovendien is (a)virulentie niet geslachtsgebonden (hoofdstuk IV).

Met behulp van de virulente en de avirulente lijn en de heterozygote F_1 in combinatie met een betrouwbare techniek om mannetjes te verzamelen werd het effect van heterozygotie op de vorming van mannetjes en vrouwtjes bestudeerd. Zowel de homozygote (AA) als de heterozygote (Aa) avirulente larven kunnen zich op cultivars met het H_1 gen niet tot vrouwtjes ontwikkelen maar wel tot mannetjes. Heterozygote avirulente mannetjes waren enigszins in het voordeel in vergelijking met homozygote avirulente mannetjes. Dit verschil is echter te klein om effect te kunnen hebben op populatie-genetisch niveau (hoofdstuk V).

Deze genetische analyse bewijst dat er een klassieke gen-om-gen relatie bestaat tussen (a)virulentie in G. rostochiensis en het H_1 resistentiegen en opent de weg voor het opsporen van het (a)virulentiegen en mogelijk ook het H_1 resistentiegen. Met behulp van "single copy" DNA probes kunnen RFLP's (Restriction Fragment Length Polymorphism) opgespoord worden die nauw gekoppeld zijn met het (a)virulentiegen. Hiertoe is de F_1 (Aa) van een virulente (aa) en avirulente (AA) teruggekruist met de virulente ouderlijn (aa). De T_1 is vervolgens opnieuw teruggekruist (T_2) met de virulente ouder om virulente lijnen te selecteren voor het H_1 resistentiegen in cultivar 'Saturna' (Figuur 1). Deze kruising gaf de gewenste 1:1 uitsplitsing te zien. Er worden nu ongeveer 300 T_2 lijnen vermeerderd. Een set discriminerende probes voor de ouderlijnen is in ontwikkeling. In het geval de RFLP's niet zijn gekoppeld met (a)virulentie, zullen in de 300 T_2 lijnen de DNA-banden van beide ouderlijnen aanwezig zijn. Geldt echter koppeling dan wordt alleen het DNA-patroon van de virulente ouderlijn verkregen, tenzij er recombinatie heeft plaatsgevonden. Het percentage recombinanten geeft waardevolle informatie voor het construeren van koppelingskaarten. Ook kan de fysische afstand tussen een aantal RFLP's die nauw gekoppeld zijn met het (a)virulentiegen worden bepaald met behulp van "Pulsed Field Electrophoresis". Dit is een belangrijke

stap op weg naar het isoleren en karakteriseren van dit (a)virulentiegen in het aardappelcysteaaltje, wat vervolgens kan leiden tot localisatie van het complementaire resistentiegen in de plant.



Figuur 1. Kruisingen voor het bepalen van de mate van koppeling tussen de markers (RFLP's) en het (a)virulentiegen in Globodera rostochiensis voor het H_1 resistentiegen.

Tot slot, het hier samengevatte onderzoek betreft een viertal methodieken die ondanks hun eenvoud baanbrekend zijn geweest voor het uitvoeren van genetisch onderzoek aan aardappelcysteaaltjes: (i) het opkweken van vier tot vijf generaties aardappelcysteaaltjes per jaar, (ii) een accurate methode voor het bepalen van het aantal virulente individuen in een populatie, (iii) het kweken van cysten op aardappelwortels in Petrischalen, en (iv) het uitvoeren van gecontroleerde kruisingen tussen één vrouwtje en één mannetje. Dit veredelingswerk resulteerde in de selectie van een avirulente en een virulente lijn met behulp waarvan het genetisch bewijs werd geleverd voor het bestaan van een gen-om-gen relatie tussen (a)virulentie in G. rostochiensis en het H_1 resistentiegen.

CURRICULUM VITAE

Schrijver dezes werd op 18 januari 1953 te Utrecht geboren. In 1971 behaalde hij het HBS-B diploma aan de Openbare Scholengemeenschap Hendrik van de Vlist te Utrecht. In datzelfde jaar begon hij zijn studie aan de Landbouwuniversiteit te Wageningen. In 1974 werd het kandidaatsexamen Plantenveredeling behaald en in 1981 studeerde hij af met plantenveredeling en erfelijkheidsleer als hoofdvak en landbouwplantenteelt als bijvak.

Vanaf augustus 1981 werd gedurende drie jaar in deeltijd bij de sectie Fysiologie van de vakgroep Nematologie een "pilot-study" verricht naar de mogelijkheden van genetisch onderzoek aan het aardappelcysteaaltje. Dit vooronderzoek resulteerde in een vervolgproject van vijf jaar.

Hij is vanaf oktober 1989 als nematoloog/entomoloog verbonden aan het Instituut voor Planteziektenkundig Onderzoek (IPO) te Wageningen bij de sectie Resistentie.