

Assessing intra-specific variations in virulence in *Globodera rostochiensis* and *G. pallida*

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SUMMARY

The accuracy of the method of Mugniéry 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.

RÉSUMÉ

Estimation des variations intraspécifiques de la virulence chez Globodera rostochiensis et G. pallida

La méthode d'élevage des nématodes à kyste sur pommes de terre cultivées en boîte de Petri (Mugniéry & Person, 1976) est utilisée pour estimer le pourcentage de phénotypes virulents et non virulents dans des populations de *Globodera*. Plusieurs populations de divers pathotypes de *G. rostochiensis* et *G. pallida* sont étudiées. Les fréquences des proportions de pathotypes virulents notées pour chaque population sont comparées à celles obtenues à partir d'essais en pots utilisant le rapport P_f/P_i .

Currently eight pathotypes are recognized in Europe by the international pathotype scheme, five within *Globodera 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 Mugniéry 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 Mugniéry 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 Mugniéry and Person (1976) and Mugniéry (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. & Buk., *S. vernei* Bittm. & 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 Ro₅-Harmerz (Ro₅-H) and Pa₃-Frensweigen were supplied by Dr. H. J. Rumpfenhorst, Münster, FRG, population Pa₂-HPL₁ 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, Bakker & Gommers, 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_i/P₁ on a susceptible clone.

Four sets of experiments were carried out :

Experiment 1 - Petri dish/cv. Eigenheimer/Ro₁-Mierenbos. Five replicates (200 larvae/replicate); inoculum density : one to ten larvae/root tip.

Experiment 2 - Petri dish/cv. Eigenheimer/Ro₅-Harmerz and cv. Saturna/Ro₃-C₁₃₃. Five to eleven replicates; inoculum density : two larvae/root tip; inoculum size : 25 to 500 root tips.

Experiment 3 - Petri dish/cultivars Eigenheimer and Saturna/five Ro₃ populations. Ten replicates; inoculum density : two larvae/root tip; inoculum size : 50 and 75 root tips for respectively cv. Eigenheimer and cv. Saturna.

-Pots/cv. Eigenheimer and cv. Saturna/five Ro₃ populations. Ten replicates; inoculum : 25 cysts/pot of 700 ml.

Experiment 4 - Petri dish/cvs 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/cvs Saturna and Darwina/Ro₁-Mierenbos; ten replicates; inoculum : 250 cysts/pot of 1.5 l.

Results

INOCULUM DENSITY (EXPERIMENT 1) AND INOCULUM SIZE (EXPERIMENT 2)

Table 1

Decrease of the numbers of females of Ro₁-Mierenbos on the susceptible cv. 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 : Ro₅-Harmerz on cv. Eigenheimer and Ro₃-C₁₃₃ on cv. 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.

Numbers of larvae inoculated per root tip were negatively correlated with those that developed into females (Tab. 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 five or ten 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 favours the accuracy of the test (Tab. 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 Ro₃ on cv. Saturna (Tab. 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 Mugniéry and Person (1976) with pot tests in estimating numbers of virulent phenotypes in five populations of pathotype Ro₃. The estimates of virulent phenotypes with the Petri dish are consistently higher than those from 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.

Table 3

Fraction of virulent phenotypes (\bar{v}_p) with SD and CV of five Ro₃-populations for cv. Saturna calculated as percentages of the number of females that developed on the susceptible cv. Eigenheimer.

		Ro ₃ -populations				
		C ₁₂₉	C ₁₇₆	C ₁₅₀	C ₁₅₆	C ₁₆₃
Petri dish Experiment (1)	\bar{v}_p	30.19 (2)	1.54 (2)	14.56 (2)	23.44 (2)	27.97 (2)
	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)	\bar{v}_p	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) Ten replicates.

(2) Inoculum size of 50 root tips for cv. Eigenheimer and 75 for cv. Saturna; two larvae inoculated per root tip.

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 (Tab. 4). The number of virulent

phenotypes for the differential clones vary per population. For example, populations Ro₃-C₁₃₃, Ro₅-H, Pa₂-HPL₁ and Pa₃-E₁₂₀₂ [all four of pathotypes able to overcome the resistance of the gene H₁ from *S. tuberosum* ssp. *andigena* (Toxopeus & Huijsman, 1953) in cv. Saturna] differ considerably in their frequencies of virulent phenotypes. Population Ro₃-C₁₃₃ has only 5 % virulent phenotypes whereas these percentages range from 84 to 100 in the other populations tested.

Population Ro₁-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 ten 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 Ro₃-C₁₃₃ as no cysts were found in Petri dish or pot tests.

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 cv. Eigenheimer. The SD is given in brackets.

Pathotype population	Differentials (Cultivars)		
	<i>S. tuberosum</i> spp. <i>andigena</i> (cv. Saturna)	<i>S. vernei</i> hybr. 58.1642/4 (cv. Mara)	<i>S. vernei</i> hybr. 62.33.3 (cv. 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.

Discussion

The method of Mugniéry and Person (1976) has several advantages compared to pot tests or experiments in closed containers (Foot, 1977; Phillips, Forrest & Wilson, 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; Raw-

sthorne & 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 (Tabs 2, 3). On the basis of standard deviations we used 100 root tips (200 larvae) in susceptible and 500 root tips (1 000 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. Tab. 4).

The observation that the Ro₃ population scored considerably lower when tested in pot experiments than in Petri dishes (Tab. 3) is probably due to the differences in the root systems in pots. The root system of the cv. Saturna with the H₁ resistance gene was consistently less developed than that of the susceptible cv. Eigenheimer. This disparity may differentially affect multiplication rates by factors not related to the H₁ 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 (Tab. 1). These observations confirm those of Mugniéry 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 of physiological conditions (Trudgill, 1967; Mugniéry & Fayet, 1981, 1984; Mugniéry, 1982b, 1985). 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 cv. Eigenheimer were in accordance with the maximum female production obtained by Mugniéry (1985).

The estimates of virulent phenotypes in five Ro₃ populations (Tab. 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 inter-population 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. Greet & Firth, 1977; Bakker & Gommers, 1982; Stegemann, Francksen & Rumpfenhorst, 1982; Wharton, Storey & Fox, 1983; Fox & Atkinson, 1984; Ohms & Heinicke, 1985). 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, Parrott & Ross (1967) suggested that the resistance conferred by the H₁ 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 H₁ 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 H₁ 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 H₁ 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 H₁ 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 Tables 3 and 4 this condition is not matched with either of the two species. Only *G. pallida* population Pa₂-HPL₁ and Pa₃-E₂₁₀₂ 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. & GOMMERS, F. J. (1982). Differentiation 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.
- 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.

- 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* × *S. vernei* hybrids on hatching of the potato cyst nematode *Globodera pallida*. *Ann. appl. Biol.*, 104 : 521-926.
- 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.
- MUGNIÉRY, 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*, Lima, Peru, CIP : 137-138.
- MUGNIÉRY, D. (1982b). Influence de l'hôte sur le développement et l'expression du sexe chez un nématode phytoparasite à déterminisme sexuel épigénétique, *Globodera pallida* Stone. *C. r. hebdomadaire Séances Acad. Sci., Paris*, 294, Sér. III : 49-52.
- MUGNIÉRY, D. (1985). Résistance des Solanacées à *Globodera* et influence de certains facteurs sur les résultats des tests de résistance. *EPPO Bull.*, 15 : 199-206.
- MUGNIÉRY, D. & FAYET, G. (1981). Détermination du sexe chez *Globodera pallida* Stone. *Revue Nématol.*, 4 : 41-45.
- MUGNIÉRY, 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.
- MUGNIÉRY, D. & PERSON, F. (1976). Méthode d'élevage 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. appl. 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 zweidimensionale Elektrophoresen ihrer Proteine. *Nachrichtenb. D. 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.
- TRIANAPHYLLOU, A. C. (1987). Genetics of nematode parasitism on plants. In : Veech, J. A. & Dickson, D. W. (Eds). *Vistas on Nematology*, Hyattsville, MD, USA, Society of Nematologists : 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*. New York, Academic Press : 235-248.

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