Epidemiology of rhizomania disease of sugar beet

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Epidemiology of rhizomania disease of sugar beet

Epidemiologie van rhizomanie bij suikerbiet

Proefschrift

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Stellingen

1. De mate van besmetting van grond met een door een schimmel overgebracht virus kan geschat worden met behulp van grondverdunningen en een biotoets.

2. De epidemische ontwikkeling van rhizomanie in een bietegewas, zoals waargenomen aan de symptomen, is een monocyclisch proces, terwijl de epidemie van infectieuze eenheden van het virus in het wortelstelsel polycyclisch is.
   Dit proefschrift.

3. Op de lange duur gaat het gebruik van suikerbietrassen met resistentie tegen het rhizomanievirus gepaard met een afname in de populatie virusbevattende rustsporen van de vector in de grond.
   Dit proefschrift.

4. Kwantitatief-epidemiologische studies over door bodemschimmels overgebrachte virussen zijn schaars vanwege het tijdrovende karakter van detectie en kwantificering van virus en vector in grond.
   7th International workshop on epidemiology of plant diseases, Arnhem 10-15 april 1994.

5. Voor epidemiologisch onderzoek aan rhizomanie heeft inoculatie van biet volgens de methode van Harveson & Rush (1993) als nadeel dat het inoculum niet gekwantificeerd is en derhalve de vermeerdering van het inoculum niet te bepalen is.
   R.M. Harveson & C.M. Rush, 1993. Phytopathology 83: 1216-1219.

6. De waardplanttoetsing door Ratna et al. (1991) geeft onvoldoende aanleiding voor de veronderstelling dat *Polymyxa graminis* ook biet zou infecteren.
   A.S. Ratna et al., 1991. Annals of Applied Biology 118: 71-78.

7. Voor navolging van een toetsmethode op resistentie van biet tegen rhizomanie is karakterisering van het inoculum noodzakelijk, dit in tegenstelling tot de bewering van Paul (1993).
   H. Paul. Proefschrift LUW, 1993. 115 blz, p. 91.

8. Het naijlen in de verschijning van een wetenschappelijk tijdschrift mag niet leiden tot het opnemen van artikelen die blijkens de datering van de jaargang een jaar eerder worden gepubliceerd dan ze zijn ontvangen en geaccepteerd.
Parasitica 47 (1991), 46 (1990), 45 (1989).

9. Ziektewerende eigenschappen van GFT-compost kunnen een meerwaarde geven aan dit substraat bij toepassing in potgrondmengsels in de boomkwekerij.

10. Om een ziektewerend effect van GFT-compost aan te tonen is het weinig reëel om gepasteuriseerd of gesteriliseerd zand als basissubstraat te nemen.
C. Schüler et al., 1989. Agriculture, Ecosystems and Environment 27: 477-482. C. Schüler et al., 1989. Journal of Phytopathology 127: 227-238. C. Schüler et al., 1993. Biological Agriculture and Horticulture 9: 353-360.

11. Met voorstellen over het weren van vluchtelingen op basis van hun werelddeel van herkomst lijkt een liberale partij uit te zijn op electoraal gewin in een niet-liberale hoek.

12. Niet alles van waarde behoeft studiepunten.
Wagenings Universiteitsblad 15, 21 april 1994, p. 7.

13. Bij een verzoek om niet te roken blijkt dat de smeulende rookwaar van sommige rokers een lont in een kruitvat is.

14. Hardlopen doe je vanuit je hart en niet op basis van je hartslagmeter.

15. Originaliteit blijkt geen voorwaarde te zijn voor citatie van een stelling in het NRC Handelsblad.

Stellingen behorend bij het proefschrift 'Epidemiology of rhizomania disease of sugar beet', te verdedigen op 16 juni 1994 te Wageningen.

Gerrie Tuitert

Author's abstract

Tuitert, G., 1994. Epidemiology of rhizomania disease of sugar beet. PhD Thesis, Wageningen Agricultural University, the Netherlands, VIII + 168 pp., 36 tables, 24 figures, English + Dutch summary.

Rhizomania disease of sugar beet is caused by beet necrotic yellow vein virus (BNYVV). The virus is transmitted by the soil-borne fungus *Polymyxa betae*. The disease can cause severe losses in sugar yield, depending on the level of infestation in the soil, the environmental conditions during the growing season and the susceptibility of the beet cultivar. Several aspects of the epidemiology of the disease were studied. A quantitative bioassay was developed to assess inoculum potentials of virus and vector in soil. The bioassay allowed to estimate most probable numbers (MPN) of infective units of *P. betae* with or without virus from the incidence of infected bait plants in a dilution series of infested soil. The recovery of *P. betae* by bioassay, the effect of duration of the bioassay on detection level of BNYVV and the effect of soil treatments on infectivity of viruliferous resting spores of the vector were assessed. The MPN method enabled the establishment of a nonlinear relationship between inoculum potential of BNYVV in soil before sowing and disease incidence and yield parameters at harvest in an artificially infested field. In the same field, the dynamics of pathogen and vector populations during two successive beet crops in the absence or presence of drip irrigation was studied. A rapid increase of inoculum of BNYVV was found and, at the highest initial inoculum level (inoculum applied in 50 g infested soil m^{-2}), sugar yield was reduced by 10% in the first and by 66% in the second year. Horizontal dispersal of viruliferous inoculum and spread of disease by movement of zoospores of the vector and by root growth of the host was limited to small distances. Displacement of infested soil by tillage practices resulted in spread over larger distances. Newly formed resting spores in roots of BNYVV-resistant plants were less viruliferous than those formed in roots of susceptible plants. A high level of BNYVV-resistance will be needed to reduce the build-up of virus inoculum in the field, which will contribute to the durability of disease resistance.

Keywords: beet necrotic yellow vein virus, *Polymyxa betae*, *Beta vulgaris*, detection, most probable number method, bioassay, resting spores, virus vector, transmission, spore germination, dispersal, spread, population dynamics, inoculum potential, inoculum density - yield relation, viruliferous population, resistance

Contents

Chapter 1

Introduction

Introduction

Rhizomania disease of sugar beet (*Beta vulgaris*) is caused by beet necrotic yellow vein virus (BNYVV) (Tamada and Baba, 1973). The virus is transmitted to the roots of the plant by the soil-borne fungus *Polymyxa betae* (Keskin, 1964; Fujisawa and Sugimoto, 1977). The common name of the disease ('root madness') is derived from the root beard'ng symptom, caused by a proliferation of lateral rootlets. Above-ground symptoms are less specific; pale green leaves, often elongated and asymmetrical, with an upright posture. Leaves with typical yellow or necrotic veins are seldom observed and only occur when the virus is transported to the leaves. Rhizomania can cause a reduction of sugar content and root weight, which can result in sugar yield reductions of up to 80%. The host range of the virus is restricted to *Beta* spp. and spinach.

The disease was first described in Italy (Canova, 1959), and later in Japan (Masuda et al., 1969). It is now widely distributed over most sugar beet growing areas in Europe (Asher, 1993) and the USA (Harveson and Rush, 1993). The occurrence in the Netherlands was confirmed in 1983 (Heijbroek, 1989).

The virus survives in thick-walled resting spores of the vector. Both vector and virus can survive in soil for 15 years or more (Abe and Tamada, 1986). As a consequence of the slow decline of inoculum potential in soil (Schlösser, 1988), crop rotation adjustments cannot be applied as a control measure. A longer rotation can only help to slow down the increase in infestation level. The damage by rhizomania can be reduced somewhat by agronomic measures that delay the time of initial infection to a later growth stage of the plant, e.g. early sowing (Ahrens, 1986) and transplanting of beet (Abe, 1987). Transplanting is too costly in European sugar beet growing, but it is applied in Japan (Abe, 1987). Soil disinfection was shown to increase yield (Schäufele, 1987), but is not an environmentally and economically desirable option for disease control. A number of chemical compounds were tested for their activity against the vector, but none effectively prevented infection of plants in the field (Schäufele, 1987; Asher, 1988). Biological control has received little attention. *Trichoderma harzianum* was shown to infect resting spores of *P. betae* in laboratory experiments (D'Ambra and Mutto, 1986) and to reduce the severity of infection of beet roots by *P. betae* in naturally infested soil in greenhouse assays (D'Ambra et al., 1987; Camporota et al., 1988). However, as with other measures that result in a decrease of inoculum of the vector, the efficacy will be limited because the virus can still be transmitted by the fraction of resting spores that survive. The most promising solution for effective control of the disease is by breeding resistant cultivars (Lewellen et al., 1987).

Upon the detection of rhizomania in the Netherlands, the Sugar Beet Research Institute (IRS) at Bergen op Zoom initiated investigations on the dissemination of the disease (Heijbroek, 1988), on effects of cultural measures, on soil disinfection and on the available 'rhizomania-tolerant' sugar beet lines that were screened in field trials. In addition, diagnostic services were rendered to the Dutch sugar companies.

As effective control measures were not available, rhizomania posed a threat to sugar beet growing in the Netherlands and its presence endangered export of planting material (e.g. seed potatoes) with adhering soil to countries presumed to be free of the disease (Hill, 1989; Asher, 1993). Many gaps in the knowledge of the epidemiology of this pathosystem had still to be filled. To stimulate research on this new and menacing disease, the Ministry of Agriculture, Nature Management and Fisheries rewarded several research proposals from various institutions, from 1987 onwards. Research on

screening methods for breeding for resistance was started at the DLO Centre for Plant Breeding and Reproduction Research (CPRO-DLO, Wageningen). The translocation and distribution of the virus within the plant was studied at the Department of Virology (Wageningen Agricultural University). Soil moisture requirements of the vector and effects of root exudates of different crops on germination of resting spores were studied at the DLO Research Institute for Plant Protection (IPO-DLO, Wageningen). Research on the prevention of spread by phytosanitary measures and on soil and plant sampling patterns in the field were performed at the Research Station for Arable Farming and Field Production of Vegetables (PAGV, Lelystad).

At the IRS, I started to work in 1987 on a broadly formulated project 'Diagnostics and epidemiology of rhizomania', an addition to the existing research programme at the same institute. The research performed from 1987 to 1993 was focused on the study of the inoculum density - disease relationship, the dynamics of viruliferous inoculum in soil and the dispersal of inoculum. In the present thesis the major results are described.

Diagnostics, the recognition of diseases from symptoms, was not an urgent topic. A more appropriate research item to include in my project was 'detection'. As the virus is soil-borne and neither virus nor fungus could be detected directly in soil, detection of either one in soil depended on their detection in host plants growing in the soil. Assessment of the presence of BNYVV in a soil sample by means of a bioassay presented qualitative information only, therefore I investigated the possibilities of obtaining a quantitative measure of the infestation of a soil sample by means of a practicable method. In Chapter 2, a quantitative bioassay is described. Next, some factors of importance to detection of virus and vector in soil were studied; the recovery of resting spores of *P. betae* from soil and the effect of the duration of the bioassay on the detection level of BNYVV in Chapter 3, the effect of conditions during storage of infested soil on infectivity of resting spores in Chapter 4.

Chapters 5 to 8 form the major section of the thesis and deal with the epidemiology of rhizomania. Epidemiology is the study of disease in populations (Van der Plank, 1963). Plant disease epidemiology is concerned with the changes, in time and space, of populations of pathogens in populations of plants. The relation between soil-borne inoculum and subsequent disease development, recorded as incidence of BNYVV-infected plants and as yield reductions obtained, is reported in Chapter 5. Temporal changes in populations of viruliferous and non-viruliferous vector in soil, as influenced by the growing of two successive beet crops in the presence or absence of irrigation, were studied by means of the quantitative bioassay. These population measurements are dealt with in Chapter 6. Spatial aspects of an epidemic were also paid attention to. Spread of the disease can occur by the different ways in which infested substrates, e.g. plant material, soil, manure or water, can be displaced. Dispersal, the movement of propagules from infected plant tissue or plants to healthy tissue or plants (Van der Plank, 1967), of BNYVV mainly depends on dispersal of the vector. Horizontal spread of BNYVV in soil, involving dispersal of viruliferous *P. betae*, extension of the infested area by root growth, and displacement of infested soil, is described in Chapter 7. The efforts of breeding companies have resulted in several partially resistant cultivars. The question as to what the effect of partial resistance of the plant to BNYVV may be on the level of viruliferous inoculum in soil, is addressed in Chapter 8. A general discussion in Chapter 9 concludes the thesis.

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Chapter 2

Assessment of the inoculum potential of *Polymyxa betae* and beet necrotic yellow vein virus (BYNVV) in soil using the most probable number method

Neth. J. Pl. Path. 96 (1990) 331-341

Assessment of the inoculum potential of Polymyxa betae and beet necrotic yellow vein virus (BNYVV) in soil using the most probable number method

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Abstract

Application of a bioassay on serial dilutions of rhizomania-infested soil provided adequate information on the level of infestation with *Polymyxa betae* and beet necrotic yellow vein virus (BNYVV).

Different combinations of dilution ratios and numbers of replicates (N) that had the same average precision were compared. A most probable number (MPN) computer programme was written to enable the comparison, because MPN tables available in literature are limited to certain dilution ratios and values of N.

Most probable numbers of infective units per ml soil assessed for infested soil from the Noordoostpolder and from Tholen (the Netherlands) were 48 for *P. betae* with 7.1 for BNYVV and 16 for *P. betae* with 1.6 for BNYVV, respectively. So in these soils 10-15% of the infective population of *P. betae* was viruliferous.

The inoculum potential of stored soil samples was not affected by conditions during storage for 28 months (dry and warm or wet and cool).

Additional keywords: rhizomania, detection, bioassay, bait plant, MPN, dilution method, quantification, infestation level, viruliferous population, storage of soil, storage conditions, sugar-beet.

Introduction

Polymyxa betae (Keskin, 1964), Plasmodiophoraceae, is the vector of beet necrotic yellow vein virus (BNYVV) (Tamada, 1975; Fujisawa and Sugimoto, 1976; Giunchedi and Langenberg, 1982) which causes rhizomania of sugar-beet. This disease reduces sugar content and yield of beet. The reduction depends on the level of infestation with viruliferous *P. betae* in the soil, soil moisture, soil temperature and the sensitivity of the beet cultivar.

Detection of *P. betae*, an obligate root parasite, in soil is possible by a bioassay using bait plants. For BNYVV there is no reliable method either for directly testing the infestation of the soil. Different procedures for bioassays are being used in rhizomania research (Merz and Häni, 1985; Beemster and De Heij, 1987; Heijbroek, 1988; Hofmeester and Tuitert, 1989). The bioassays provide information on the presence or absence of *P. betae* and/or BNYVV. For evaluation of levels of infestation and the relationship with disease incidence and yield of sugar-beet a quantitative estimation of the infective inoculum is needed.

Quantification is possible with a bioassay when serial dilutions of the soil are used. In aliquots of the different dilutions the presence (positive) or absence (negative) of a microorganism is determined. The series of numbers of positives per dilution is related to the number of infective units of the organism in the original sample. The principles of what is called the dilution method or most probable number (MPN) method are described in detail by Halvorson and Ziegler (1993), Cochran (1950) and Kleczkowski (1968). The method was originally used for enumerating bacterial populations in various substrates (Halvorson and Ziegler, 1933). It has been applied on bioassays or plant infection techniques for estimation of populations of fungal plant pathogens (Maloy and Alexander, 1958; Hornby, 1969; Duncan, 1976; Pfender et al., 1981), mycorrhizal fungi (Porter, 1979) and rhizobia (Scott and Porter, 1986) in soil.

Studies on methods for assessment of the population density of *P. betae* in soil are in progress in various countries, but BNYVV is not included in these methods (Asher and Blunt, 1987; Goffart et al., 1987; Ciafardini and Marotta, 1989). The percentage of BNYVV-infected bait plants in a bioassay can give an indication of the infestation of soil (Beemster and De Heij, 1987; Hofmeester and Tuitert, 1989).

Since BNYVV is responsible for rhizomania, determination of levels of infestation by quantitative estimation of the viruliferous *P. betae* population is required. Therefore, the MPN method should also give reliable results for the assessment of BNYVV.

In this article the application of the MPN method for assessment of both vector and virus is described. Different dilution ratios are compared and different numbers of replicates are used with a practicable bioassay method. The method was used to investigate the effect of different storage conditions of soil samples on subsequent detection of *P. betae* and BNYVV.

Materials and methods

MPN method. The application of the theory of probability for the numerical interpretation of the dilution data was performed by various workers, who presented tables of MPNs (Buchanan and Fulmer, 1928; Halvorson and Ziegler, 1933; De Man, 1975; Anonymus, 1985). These tables are usually based on 10-fold dilutions, with a fixed number of replicates per dilution. Fisher and Yates' method uses only 88% of the information obtained in the experiment (Fisher and Yates, 1963). Halvorson and Ziegler (1933), Cochran (1950) and Kleczkowski (1968) described the maximum likelihood equations for calculation of the MPN. Parnow (1972) developed a computer programme for this calculation.

The data presented in this paper were analyzed by a computer programme written in Fortran, based on equations given in the last four references. The programme calculates the MPN and confidence limits of the estimate, according to Cochran (1950), for any number of dilution levels and any combination of replicates per dilution. The calculation was checked by comparison of the MPN values with values from existing tables.

Cochran (1950) described the steps in planning a dilution series. The choice of the dilution ratio and the number of replicates is determined by the desired precision and the amount of work that is practicable to do, considering that the method should be applied in routine assessments.

The average precision of the MPN method is nearly the same for any dilution ratio

Table 1. Details of dilution series tested.

Dilution ratio	Number of test plants per dilution (N)	Total number of test plants	Standard error of $^{10}\log d^1$
5	7	7 × 7 = 49	0.174
10	10	5 × 10 = 50	0.183
5	4	7 × 4 = 28	0.230
10	6	5 × 6 = 30	0.237

[1] d = estimated density or most probable number (MPN) (Cochran, 1950).

between 2 and 10, if the total number of samples in the whole series is kept fixed (Cochran, 1950).

From previous work (unpublished) it was deduced that the lowest dilution of a soil sample should be 10^{-5}. Within this range the number of dilutions required for the dilution ratios 5 and 10 is seven and five respectively. Results of dilution series with a different dilution ratio and with different numbers of replicates should yield the same MPN.

Details of the series that were compared are given in Table 1.

Soil sample preparation and serial dilution. Rhizomania-infested soil was collected from the upper 15 cm of a field in the Noordoostpolder (NOP), the Netherlands. The soil was air-dried, mixed thoroughly and ground with pestle and mortar. Samples of the infested soil were serially diluted by volume with coarse (sieved over 2 mm sieve) sterilized dry sand. At every dilution step the soil and sand were thoroughly mixed by vigorous shaking in an inflated plastic bag before collecting a composite sub-sample for the next dilution.

Bioassay. The soil-sand samples were humidified with a small spray gun. Per dilution, the required number of pots were filled with 200 ml of the mixture. In each pot a sugar-beet seedling (cv. Regina, 2-3 weeks old) was planted. The pots were placed in a greenhouse with a day and night temperature of 23 and 15 °C, respectively. Each pot was placed in a Petri dish (diameter 9 cm). Water, and once a week Steiner nutrient solution, was added regularly so as to keep the soil moist. The plants were analyzed for presence of *P. betae* and BNYVV after an incubation period of six weeks.

Plant analysis. After removal of the pot, the soil-sand mixture around the roots was washed away with running tap water. Part of the rootlets was removed and examined for *P. betae* at ×200 with a light microscope (Olympus inverted microscope CK2). Sap was extracted with a handpress from the rest of the rootlets and the tap root. One hundred µl sap was diluted with 900 µl extraction buffer.

The presence of BNYVV was tested by double antibody sandwich ELISA as described by Clark and Adams (1977). Slight modifications in the procedure were applied. Wells were coated with 100 µl coating buffer. Also the amount of substrate, 2-nitrophenyl phosphate (Merck) used at 5 mg/ml in substrate buffer, was 100 µl per well. Sample

and conjugate were incubated simultaneously (Flegg and Clark, 1979; Van Vuurde and Maat, 1985); per well 100 µl of both and incubated overnight at 4 °C.

Buffers and (conjugated) antibodies from the Sanofi diagnostic kit (Sanofi Phytodiagnostics, France) were used. Results were recorded at 405 nm using a Titertek Multiskan photometer. Absorbance values exceeding 0.050 were considered to be positive. Average absorbance values for the non-infected controls were 0.020 ± 0.006 (N = 10) for the results presented in Table 4 and 0.005 ± 0.002 (N = 6) for Table 5.

Effect of storage conditions of soil samples. Rhizomania-infested soil was collected from a field in Tholen, the Netherlands. The moist soil was sieved (5 mm), mixed and stored under different conditions:

A. air-dry at room temperature (20 °C);

B. moist (pF = 2.2-2.5) and cool (5 °C).

After a storage period of 28 months both the infested soil and the sterile diluent sand were sieved (2 mm). The moist soil (B) was air-dried at 20 °C during 7 days before the assessment. The infested soil was ground with pestle and mortar. Per treatment two samples were serially diluted with dilution ratio 10, followed by the bioassay with six replicates per dilution, performed as described before.

Results

MPN values calculated for different combinations of data from three dilutions with different numbers of replicates were compared with those presented in the literature (Table 2).

The estimation of the MPN in the infested soil did not depend on the dilution series used, whether it be for *P. betae* or for BNYVV (viruliferous *P. betae*) (Tables 3 and

Table 2. Comparison of calculated MPN values with values from tables in the literature for different combinations of data from three dilutions with dilution ratio 10 and different numbers of replicates (N) per dilution.

N	Number of positives in dilution			MPN value					
	1×	0.1×	0.01×	Tuitert	Parnow[2]	De Man[2]	B & F[3]	H & Z[4]	F & Y[5]
3	2	0	0	0.92	0.92	0.9	0.9	–	0.86
3	3	3	2	109.90	109.90	110	110	–	106.80
5	0	0	1	0.18	0.18	0.2	0.2	–	0.27
5	4	0	0	1.28	1.27	1.3	1.3	–	1.14
5	5	5	4	160.90	160.94	160	160	–	113.80
10	1	2	0	0.29	–	0.29	–	0.29	0.35
10	5	3	1	1.13	–	1.1	–	1.13	1.40
10	10	9	6	39.77	–	40	–	39.8	58.07

[1] Parnow (1972); [2] De Man (1975); [3] Buchanan and Fulmer (1928); [4] Halvorson and Ziegler (1933); [5] according to Fisher and Yates' method (1963).

Table 3. Number of bait plants infected by *Polymyxa betae* (positives) at different dilution levels of infested soil and MPN estimated with dilution ratios 5 and 10, N replicates per dilution.

Dilution ratio = 5			Dilution ratio = 10		
dilution	number of positives		dilution	number of positives	
	$N = 7$	$N = 4$		$N = 10$	$N = 6$
5^{-1}	7	4	10^{-1}	10	6
5^{-2}	7	4	10^{-2}	10	6
5^{-3}	7	4	10^{-3}	10	6
5^{-4}	7	4	10^{-4}	6	4
5^{-5}	7	4	10^{-5}	0	0
5^{-6}	2	2			
5^{-7}	2	0			
MPN/ml soil[1]	53	51		40	47

[1] Values are not significantly different at $P = 0.05$ (Cochran, 1950).

4). The MPN for BNYVV was significantly lower ($P = 0.05$) than the MPN for *P. betae*. About 15% of the infective population of *P. betae* appeared to be viruliferous.

The conditions at which the soil samples were stored during a period of 28 months did not influence the results of the assessment of vector and virus, as the MPNs for *P. betae* or BNYVV were not significantly different in the two treatments (Table 5). The MPN for BNYVV was significantly lower than for *P. betae*, the viruliferous population was about 10%.

Table 4. Number of BNYVV-infected bait plants (positives) at different dilution levels of infested soil and MPN of viruliferous *Polymyxa betae* estimated with dilution ratios 5 and 10, N replicates per dilution.

Dilution ratio = 5			Dilution ratio = 10		
dilution	number of positives		dilution	number of positives	
	$N = 7$	$N = 4$		$N = 10$	$N = 6$
5^{-1}	7	4	10^{-1}	10	6
5^{-2}	7	4	10^{-2}	10	6
5^{-3}	7	4	10^{-3}	5	5
5^{-4}	7	3	10^{-4}	3	1
5^{-5}	3	2	10^{-5}	0	0
5^{-6}	0	0			
5^{-7}	0	0			
MPN/ml soil[1]	9.2	5.5		5.0	8.8

[1] Values are not significantly different at $P = 0.05$ (Cochran, 1950).

Table 5. Inoculum potential of *Polymyxa betae* and BNYVV in infested soil stored under different conditions for 28 months. Bioassay on serial dilutions of two samples per treatment, dilution ratio 10, 6 replicates per dilution.

Storage conditions	MPN/ml dry soil[1] (95% confidence limits)[2]			
	P. betae		BNYVV	
Air-dry, 20 °C	16	(5, 46)	0.9	(0.3, 2.6)
	21	(7, 64)	1.9	(0.6, 5.6)
Moist, 5 °C	12	(4, 34)	1.3	(0.5, 4.0)
	16	(5, 46)	2.1	(0.7, 6.3)
Mean MPN	16		1.6	

[1] MPN = most probable number of infective units. Values are not significantly different within and between treatments at $P = 0.05$ (Cochran, 1950).
[2] According to Cochran (1950).

Discussion

The application of the dilution method on the bioassay for detection of rhizomania in soil proved to be an adequate method for measuring the inoculum potential in the soil. For *P. betae* as well as for BNYVV (viruliferous *P. betae*) the different dilution series showed a similar range of positives, resulting in corresponding MPN values.

The conditions during storage of soil samples did not affect the level of the inoculum potential. It was neither influenced by the storage period: assessment after storage for 11 months at dry and moist conditions resulted in MPNs of 21 and 31 for *P. betae* and 2.8 and 3.1 for BNYVV, respectively (G. Tuitert, unpublished).

Values calculated with the MPN computer programme corresponded with values from tables in the literature. The programme enabled the comparison of combinations of dilution ratios and numbers of replicates that had the same average precision, because MPN tables available in literature are limited to certain values for the dilution ratio and number of replicates.

There are two principal assumptions on which the application of the theory of probability is based (Cochran, 1950). The first is that the propagules of the microorganism are distributed randomly in the medium and follow a Poisson distribution in replicate samples. The second assumption is that the presence of a single propagule certainly causes a positive reaction. This implies for a root-infecting pathogen that the soil should be thoroughly mixed, that the total volume of soil is explored by the roots, and that the conditions of the bioassay are optimal for infection and disease development. In the bioassay dilution method described here these prerequisites are met and favourable soil moisture and temperature conditions for infection according to Asher and Blunt (1987) are secured.

Probably not all the resting spores present are infective; in bioassay methods it is therefore more correct to express results in terms of infective units in stead of in those of viable units of the organism determined (Maloy and Alexander, 1958).

The incubation period should be sufficient for both development of *P. betae* and

multiplication of BNYVV to a detectable level. Resting spores of *P. betae* in soil germinate in the presence of sugar-beet roots by releasing zoospores that cause primary infection of the roots. When the density of resting spores is low, root density may be the limiting factor for infection in an early stage of the bioassay. Indeed dilution of infested soil slowed the rate of infection of the bait plants by *P. betae* (Asher and Blunt, 1987). Once primary infection has taken place and plasmodia are formed, development may follow two directions. In a rapid multiplication cycle, plasmodia differentiate into zoosporangia that release secondary zoospores from the roots. This cycle may take 40-80 hours (Keskin, 1964), so several generations of zoospores can be produced and severe infection of *P. betae* can soon be detected. In the alternative phase of development plasmodia differentiate into clusters of resting spores (cystosori). Asher and Blunt (1987) recorded the changes in percentage of plants that showed the different infection structures. Six weeks after sowing, 100% of plants in a soil with an unknown level of infestation showed mature clusters of resting spores.

An incubation period of six weeks in this bioassay will be long enough to observe *P. betae* infection, even at low dilutions, the more so as not only mature resting spores are recognizable, but also immature resting spores and the typical zoosporangia.

As for BNYVV, the situation is quite different. The viruliferous population of *P. betae* probably comprises only a part of the total infective *P. betae* population. So primary and secondary zoospores may or may not be viruliferous. There may be a competition between these zoospores and a rapid enhancement of virus concentration in the plant may be hindered. After introduction into the plant BNYVV multiplies in the infected epidermal cells and is transported within rootlets and to a lesser extent into the taproot by the xylem vessels (Giunchedi and Poggi Pollini, 1988). However, cell-to-cell movement of BNYVV depends to a great extent on the secondary infection cycle of the vector during the early stages of plant growth and infection (Hillmann and Schlösser, 1986).

Gerik and Duffus (1988) observed that cultures of viruliferous *P. betae* showed a higher incidence of root infection than nonviruliferous isolates, which indicates that a viruliferous population might outcompete a nonviruliferous population. This is a preliminary assumption, since it is not known whether the observation was due to the effect of the virus on the vector or the effect of the virus on the plant (or an interaction). Moreover, information on incidence of root infection after using mixed populations of both viruliferous and nonviruliferous *P. betae* was not provided.

It has been demonstrated that nonviruliferous isolates are able to acquire and transmit BNYVV (Abe and Tamada, 1986; Gerik and Duffus, 1988). Therefore, competition might not be that important, and may occur only when the viruliferous population is a very small fraction of the total population of *P. betae*.

Still, the incubation period is important to ascertain that the virus, once introduced into the plant, will multiply to a detectable level. Improbable results in the range of positive values in a dilution series (De Man, 1975), although not obtained in the experiment described, are more likely to occur for BNYVV than for the fungus. First, especially at low inoculum densities, primary infection may take place in a late stage. Infection time and site will probably vary for the replicates of these densities, so the detection level in the ELISA may or may not be reached. Secondly, there are the constraints of the ELISA, considering detection level and the positive/negative threshold (Sutula et al., 1986). Thirdly, the genetical heterogeneity within the used sugar-beet genotype can

cause differences in the concentration of the virus (Giunchedi et al., 1987).

These considerations hold for all low density dilutions of soil tested. So results, although perhaps not really quantitative, do give information to compare and judge the inoculum potential of rhizomania in soils.

Goffart et al. (1987) and Ciafardini and Marotta (1989) applied the dilution method to quantify *P. betae*, but did not include BNYVV in their method. The bioassay procedure of Goffart et al. (1987) includes two weeks of water-saturated conditions, which might cause the interference of *Aphanomyces cochlioides* in the test, as described. The method seems to be less practicle for routine use, also the total number of test plants (70) needed to assess one soil sample is high. Ciafardini and Marotta (1989) made dilutions of suspensions of the soil. The soil sample tested is small and the method seems to be rather laborious, but it has the advantage that it requires only a small amount of space.

Blunt and Asher (1989) also gave quantitative data for *P. betae* obtained by application of a dilution method. Their method differed from the described procedure in the amount of soil per pot, the dilution ratio and the large total number of plants to be tested (S.J. Blunt, personal communication).

Beemster and De Heij (1987) described a method for detection of both *P. betae* and BNYVV and mentioned the possibility of obtaining quantitative data. After exposure to soil for four days the bait plants were transplanted in sterile sand. Assessment of the percentage of infected plants allowed an estimation of the level of infestation of the soil. A disadvantage of this method is that the quantitative estimation is not based on independent observations; all plants are incubated in the same Petri dish. Also the results might be influenced by the number of plants used. Besides, since after baiting for 24 hours infected plants can already be detected (G. Tuitert, unpublished) and considering the period of 40-80 hours required for the multiplication cycle (Keskin, 1964) the length of the baiting period (four days) does not exclude the possibility that secundary infection takes place.

The quantitative method presented here is the first application of the dilution method on a bioassay for a vector-transmitted virus. It estimates the inoculum potential of the pathogen rather than the inoculum density (Baker, 1965; Bouhot, 1979; Mitchell, 1979).

The mean MPN values obtained for *P. betae*, 48 per ml soil from the Noordoostpolder and 16 per ml for soil from Tholen, are higher than values reported for Belgian and English soils (Goffart et al., 1987; Blunt and Asher, 1989), but are in the same range as that for soil from northern Italy (Ciafardini and Marotta, 1989).

Both soil samples analyzed were collected from fields known to be heavily infested with rhizomania. For BNYVV the mean MPNs were 7.1 and 1.6 per ml soil for NOP and Tholen respectively. In these soils the viruliferous infective *P. betae* population seemed to be about 10-15% of the total infective population, assuming that the rate of germination and infection is the same for viruliferous and nonviruliferous *P. betae*.

For practical application of the method a small number of replicates (N) per dilution and a high dilution ratio (F), which reduces the required number of dilutions within the necessary dilution range, are preferred. The practicability of the method makes the increase in standard error and the diminished possibilities of statistical discrimination with small F and N values acceptable. Use of dilution ratio 10 with 6 replicates per dilution can thus be recommended for assessment of the inoculum potential of both vector and virus.

The method can be a useful tool in rhizomania research:

a) to study the spread of rhizomania within and between fields, and the behaviour of a viruliferous population in a predominantly nonviruliferous population of *P. betae*;
b) to quantify the risks of ways of spreading the disease;
c) to study effects of different factors on ecology and epidemiology of the disease (e.g. multiplication of vector and virus after growing one beet crop);
d) to characterize and standardize test soil used for selection of rhizomania-resistant or -tolerant plants.

Preliminary results of application of the method to quantify levels of infestation in the field indicate a relationship between MPNs determined and sugar yield of beet.

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Samenvatting

Kwantitatieve detectie van Polymyxa betae en het rhizomanievirus (BNYVV) in grond

Toepassing van de verdunningsmethode en de 'MPN'-berekening op de biotoets voor rhizomanie resulteerde in kwantitatieve gegevens over de mate van besmetting van de grond, zowel voor *P. betae* als voor BNYVV.

De besmettingsgraad van bewaarde grondmonsters werd niet beïnvloed door de bewaaromstandigheden gedurende 28 maanden (droog en warm of vochtig en koud).

Mogelijkheden voor praktische toepassing van de methodiek worden besproken.

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Chapter 3

Recovery of resting spores of *Polymyxa betae* from soil and the influence of duration of the bioassay on the detection level of beet necrotic yellow vein virus in soil

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Recovery of resting spores of Polymyxa betae from soil and the influence of duration of the bioassay on the detection level of beet necrotic yellow vein virus in soil

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Abstract

In a bioassay, the recovery of resting spore clusters of *Polymyxa betae* from artificially infested sand was 1.7%. A recovery between 1 and 2% implies that one infective unit of *P. betae* estimated with the most probable number method represented 50–100 resting spore clusters.

Neither prolongation of the duration of the bioassay from 6 to 18 weeks, nor a second and third 6-week replant resulted in a lower dilution endpoint of BNYVV in naturally infested soil. Estimates of inoculum potential of BNYVV were not significantly increased when incubation was extended over 6 weeks. In the 18-week bioassay, tap root weights showed a decrease with increasing inoculum levels. The presence of BNYVV in the tap root, as detected by ELISA, was associated with the presence of leaf symptoms and root browning.

Using the most probable number (MPN) method, assessment of vector and virus after 6 weeks yielded 3- and 16-fold higher MPNs, respectively, than when determined after 3 weeks. Prolongation of the bioassay to 12 weeks did not result in a higher MPN of BNYVV. Total and tap root fresh weight showed a decrease with increasing inoculum levels, but lateral root weight was hardly affected, after 3, 6 and 12 weeks of incubation.

Additional keywords: BNYVV, rhizomania, MPN, dilution method, bait plant, sugar beet, soil-borne pathogen.

Introduction

Infestation of soil with beet necrotic yellow vein virus (BNYVV) and its fungal vector *Polymyxa betae* is commonly assessed by means of a bioassay using sugar beet seedlings as bait plants. A quantitative assessment of inoculum of vector and virus can be achieved by using serial dilutions of the soil (Tuitert, 1990). With this procedure, the vector propagules in the soil are not counted directly, but an estimate of the infective population is obtained only. Therefore, inoculum potential is measured rather than inoculum density, and the results of the assay are presented as most probable numbers (MPNs) of infective units. It is not known which proportion of the population of resting spore clusters of *P. betae* is infective, thus what is represented by the estimated 'units'.

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The results of bioassays are influenced by various factors including the treatment of soil prior to the assay, the volume of soil explored by the roots, the duration of the assay, and the environmental conditions during the assay. With a 6-week bioassay, similar estimates were obtained for the inoculum potential of *P. betae* (and BNYVV) in soil samples stored under different conditions (Tuitert, 1990). Apparently, effects of conditions during storage of soil on the (rate of) germination of resting spores, found in experiments with short periods of exposure (Tuitert, 1993), are only temporary. In the bioassay referred to (Tuitert, 1990), a pot size was used that was practical to handle in routine assessments and that had a volume that could be fully explored by the roots within 6 weeks. Temperature conditions in the greenhouse were about 23 °C during the day and 15 °C at night. The sensitivity of detection of BNYVV by bioassay might be increased either by obtaining higher virus concentrations in the assay plants or by using a detection technique more sensitive than ELISA, or both. Modification of the bioassay by incubation at a higher temperature also during the night or by extension of the duration of the bioassay under standard greenhouse conditions might increase the concentration of BNYVV in the bait plants.

The following aspects of detection of virus and vector by bioassay were studied. First, the recovery of *P. betae* by bioassay was determined in sand to which known numbers of resting spore clusters were added. Second, the effect of the duration of the bioassay on the level of detection of BNYVV and *P. betae* was examined.

Materials and methods

Expt 1. Recovery of resting spores of P. betae by bioassay

Two resting spore suspensions (A and B) were prepared, each from the rootlets of three sugar beet plants. One of the two triplets consisted of cv. Regina (A), the other of cv. Rizor (B). These plants had grown for 8 weeks in a mixture of 5% (v/v) naturally BNYVV-infested sandy clay soil and coarse sand. Lateral rootlets were cut into pieces with a scalpel, macerated by pestle and mortar and suspended. The suspensions were sucked through Monodur gauze (50 μm mesh), to remove large root particles, and concentrated by centrifugation (Tuitert, 1993). Densities of resting spore clusters in the suspensions were determined by means of a haemocytometer. Suspensions with 15×10^2, 15×10^3 and 15×10^4 spore clusters in 50 ml demineralized water were added to 1500 ml sterile coarse sand (pH 7). Diluted healthy root sap served as a control. The densities of spore clusters created in the sand were 0, 1, 10 and 100 per ml. In both spore suspensions the average spore cluster consisted of about 37 individual resting spores. The sand was air-dried, mixed thoroughly and then stored for 10 months at room temperature. From the 1500-ml sand lots two subsamples of 150 ml were taken and both were four times diluted with sand using a dilution ratio of 10. Per dilution, six pots of 200 ml were filled and each pot was planted with one sugar beet seedling, cv. Regina. After 6 weeks in the greenhouse, the plants were examined microscopically for the presence of *P. betae* (Tuitert, 1990). The numbers of infected plants at the different dilutions were used to estimate the MPN of infective units of *P. betae* for the highest density tested. The MPN divided by the known number of added resting spore clusters yielded the percentage recovery.

Expt 2. Effect of duration of the bioassay on the lower limit of detection of BNYVV in soil

Different levels of infestation were prepared by serial dilution of BNYVV-infested soil with a mixture of sterilized soil and sand. In two experiments, Expt 2a and 2b, the dilu-

tion endpoint was determined after 6 and 18 weeks duration of the bioassay, by assessment of BNYVV in the tap root of bait plants. Besides, in Expt 2a a replanting treatment was included; the soil of the 6-week bioassay was replanted for a second and a third 6-week baiting period.

Treatment of soil. BNYVV-infested soil from the Noordoostpolder (NOP), the Netherlands, was air-dried, mixed and ground with pestle and mortar. As a diluent, a mixture of sterilized NOP soil and coarse sand was used, 50/50 % (v/v) in Expt 2a and 20/80 % (v/v) in Expt 2b. The infested soil was first 100× diluted by weight with the diluent and then three times serially diluted with a dilution ratio of 10.

Procedure. Of each dilution, 20 pots of 200 ml were filled and randomly assigned a duration of either 6 weeks or 18 weeks in a growth chamber at 22/15 °C with a photoperiod of 16 h. In each pot, one 2-week old seedling, cv. Regina, was planted. In Expt 2a, pots of the 6-week assay were replanted twice. After removal of the tap roots from the bait plants of the first planting, the remaining roots were cut up in the pot and the soil with root fragments was mixed carefully. Then, a new beet seedling was planted in each pot. After 6 weeks, a third planting was done when tap roots of the plants from the second planting were removed.

Plant analysis for BNYVV. At the end of the growing period, sap was collected from the tap root of the bait plants and analyzed for the presence of BNYVV by ELISA (Clark and Adams, 1977; Tuitert, 1990). Whole tap roots were used, except for the large tap roots at 18 weeks, of which only the lower parts were taken.

Assessment of disease symptoms. After 18 weeks, plants were examined for the presence of leaf symptoms (light-green colour, tapering and upright shape, asymmetrical leaf halves, yellow veins) and the colour (white, light-brown, dark-brown) of the rootlets. Fresh weight of tap roots was determined. In Expt 2b, aphids and mites on the leaves during the second half of the 18-week period may have influenced growth of the bait plants.

Expt 3. Effect of duration of the bioassay on quantitative assessment of BNYVV and P. betae in soil

Using the procedure as described for quantitative estimation of the inoculum of BNYVV and *P. betae* in soil (Tuitert, 1990), a shorter (3-week) and a longer (12-week) duration than the standard period were included. At the end of the incubation periods, inoculum potential was estimated and fresh weights and volumes of bait plant roots were assessed.

A sample of soil, naturally infested with BNYVV, was dried, ground and diluted 100× by volume with sterile coarse sand and twice more serially with a dilution ratio of 10. The procedure of the assay was as described before, but in this case three dilutions instead of four, and one non-infested control series were used with 30 pots per dilution, 10 for each incubation period. After 3 and 6 weeks, complete root systems were used for the analysis of BNYVV, after 12 weeks tap root tips and lateral roots were analyzed separately. The roots were examined with the microscope for the presence of *P. betae*. Fresh weight of roots was determined and volumes of the whole root system and of the tap root were measured by determination of the amount of displaced water. After 3 weeks, volumes of the little tap roots were estimated from diameter and length.

Statistical analysis

Numbers of infected plants in the dilution series were used to estimate the most probable numbers (MPN) of infective units of *P. betae* per ml of sand or soil (Expt 1 and 3) and BNYVV per g or per ml of soil (Expt 2 and 3, respectively) (Tuitert, 1990). MPNs were compared according to Cochran (1950). In Expt 1, effects of origin of spore clusters (cultivar) and of initial density of clusters on MPNs were analyzed by ANOVA of log-transformed MPNs ($\log_{10}$ MPN), using the data of the densities 10 and 100 ml^{-1} (at density 1 ml^{-1}, MPNs could not be estimated for one of the two suspensions).

The absorbance values in ELISA of the bait plants of the replanting treatment in Expt 2a were $\log_{10}$-transformed before analysis. The effect of replanting on absorbance values of BNYVV-infected bait plants was investigated by ANOVA, using the two dilutions in which all replicates were BNYVV-infected and analyzing data from the first and third planting, and from the first and second planting, separately.

The association of leaf symptoms and root discoloration with the presence of BNYVV and/or *P. betae* was analyzed using contingency tables and χ^2 tests (Steel and Torrie, 1980).

Root weights were subjected to ANOVA of a completely randomized design using GENSTAT 5 (Payne et al., 1988). In case of increasing variance with increasing means, root weight (g) or volume (ml) data (multiplied by 1000) were $\log_{10}$-transformed before analysis. Dunnett's procedure (Steel and Torrie, 1980) was applied for comparison of all treatment means with the non-infested control: one-sided for total and tap root weight, because of the common knowledge of negative effects of BNYVV on tap root weight; two-sided for lateral root weight.

Results

Expt 1. Recovery of resting spores of P. betae

The recovery of resting spore clusters of *P. betae* from sand was low; MPNs of infective units varied from 1.1 to 3.4% and were on average 1.7% (back-transformed from mean $\log_{10}$ MPNs) of the actual number of propagules (Table 1). In ANOVA of $\log_{10}$-transformed MPNs calculated to a corresponding density by multiplying the values of density 10 ml^{-1} by 10 (density 1 ml^{-1} not included, because of the absence of two replicate estimates for suspension B), the small differences were significant. MPNs were slightly lower at density 100 than at density 10 ($P < 0.05$), and higher for suspension A than for B ($P < 0.05$). The two samples of each inoculum level yielded corresponding estimates for the MPN.

Expt 2. Level of detection of BNYVV after 6 and 18 weeks duration of the bioassay

Lower limit of detection of BNYVV in soil. In Expt 2a, an 18-week duration resulted in a dilution endpoint of 10^{-5} in the bioassay (Fig. 1A). After the standard 6-week duration the endpoint was 10^{-4}. The difference was due to one infected plant only. Replanting of the pots after 6 weeks resulted in two more BNYVV-positive plants at the two lowest dilutions, but not in a different dilution endpoint. The absorbance values in ELISA of the BNYVV-positive bait plants were higher after replanting than before ($P < 0.001$). Analysis per planting time showed no significant difference between mean absorbance of BNYVV-positive plants at the two highest inoculum levels (Table 2).

In Expt 2b, one BNYVV-infected bait plant was found in dilution 10^{-5} after 6 weeks of

Table 1. Recovery of resting spore clusters of *Polymyxa betae* from artificially infested sand, at three densities of resting spore clusters, using the most probable number method (Expt 1).

Density (number of spores ml^{-1} sand)		Spore suspension A[a]			Spore suspension B[a]		
		MPN[b]	95% conf. limits[b]	Recovery (%)	MPN[b]	95% conf. limits[b]	Recovery (%)
1	a[b]	0.04	(0.01, 0.11)	3.4	0.01[c]	(0.004, 0.03)	1.2[c]
	b	0.03	(0.01, 0.09)				
10	a	0.37	(0.12, 1.10)	3.3	0.15	(0.05, 0.46)	1.4
	b	0.28	(0.10, 0.84)		0.12	(0.04, 0.34)	
100	a	1.55	(0.52, 4.6)	1.6	0.87	(0.29, 2.6)	1.1
	b	1.55	(0.52, 4.6)		1.33	(0.45, 4.0)	

[a] Two spore suspensions were used; 'A' was prepared from roots of plants cv. Regina, 'B' from those of cv. Rizor.
[b] MPN is the most probable number of infective propagules in sand with the indicated density of spore clusters. Estimates are based on serial dilutions of two samples (a and b). The confidence limits are calculated according to Cochran (1950). See text for results of ANOVA.
[c] No infection occurred in the four serial dilutions of both samples. In the undiluted density all plants were infected; a mean MPN was calculated using this density.

incubation and none after 18 weeks (Fig. 1B).

Tap roots of 18-week-old bait plants with dark-brown rootlets and leaf symptoms of rhizomania were all BNYVV-positive. Only one (3%) of the plants without leaf symptoms was BNYVV-positive.

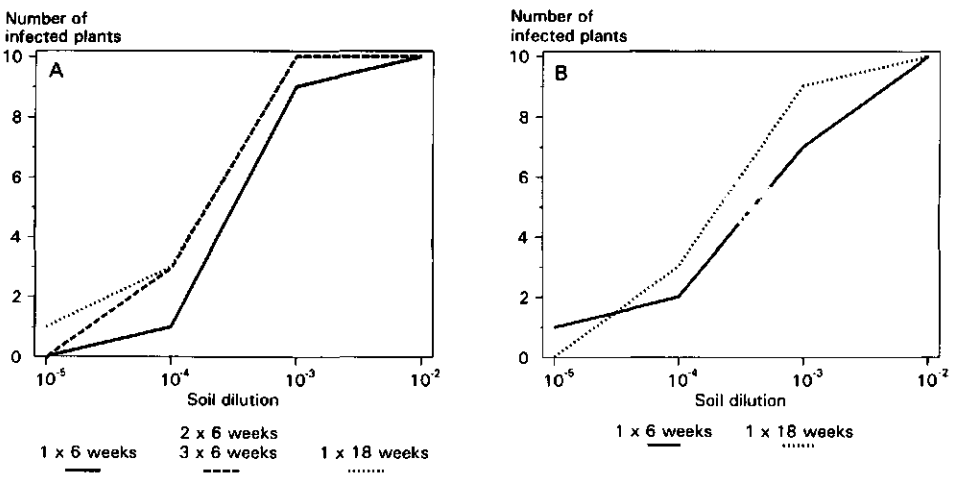

Fig. 1. Detection of BNYVV in dilutions of infested soil using a bioassay with bait plants and a duration of 6 or 18 weeks, or with replanting the 6-week pots twice. A. With replanting (Expt 2a); B. Without replanting (Expt 2b).

Table 2. Mean absorbance values[a] of BNYVV-positive bait plants in a 6-week bioassay, replanted twice for 6 weeks (Expt 2a).

Dilution of infested soil	Absorbance values of BNYVV-infected plants, multiplied by 1000[b]		
	1st planting	2nd planting	3rd planting
10^{-4}	178	423	1334
10^{-3}	173	704	1388
10^{-2}	339	893	1427

[a] Means are back-transformed from $\log_{10}$ values. Mean absorbance values for the control and other non-infected plants were 17, 16 and 5, for the three planting series, respectively.
[b] Replanting the pots of the 6-week bioassay resulted in significantly higher absorbance values for the BNYVV-infected bait plants ($P < 0.001$ for the second and third planting compared to the first, and for the third compared with the second). ANOVA was applied to the ($\log_{10}$-transformed) data from dilutions 10^{-3} and 10^{-2}. There was no significant difference ($P < 0.05$) between these dilutions.

Assessment of the inoculum potential of BNYVV in soil. The MPNs of BNYVV for both durations were not significantly different (Table 3). Replanting did not result in significantly ($P = 0.05$) higher MPNs either. Experiments 2a and 2b yielded corresponding results for either 6 or 18 weeks.

Table 3. The inoculum potential of BNYVV in soil as estimated by bioassay with different durations (Expt 2a and b). In Expt 2a, pots of the 6-week assay were replanted twice for a 6-week period (planting 2 and 3, respectively).

Planting	Duration (weeks)	Most probable numbers (MPN) of infective units of BNYVV g^{-1} soil			
		Experiment 2a		Experiment 2b	
		MPN	(95% conf. limits)[a]	MPN	(95% conf. limits)[a]
1	6	7.6	(3.3, 18)	5.2	(2.2, 12)
2	6	16	(6.7, 36)	–[b]	
3	6	16	(6.7, 36)	–	
1	18	19	(8.0, 43)	8.8	(3.8, 20)

[a] Confidence limits were calculated according to Cochran (1950).
[b] No replanting in Expt 2b.

Effect of inoculum at four levels on root weight after 18 weeks. Tap root fresh weight after 18 weeks was affected ($P < 0.001$) by inoculum (soil dilutions) in both experiments (Expt 2a and 2 b). In Expt 2a (Fig. 2A), a steep decrease in fresh weight occurred between dilution 10^{-4} and 10^{-3}; tap root weight relative to the control ranged from 78 and 49% at 10^{-5} and 10^{-4} to 6% at 10^{-3} and 10^{-2}. The effect of inoculum level is largely explained by a linear trend ($P < 0.001$).

In Expt 2b (Fig. 2B), the effect of inoculum level is again mainly explained by a linear trend ($P < 0.001$). Comparison of treatment means with the control showes lower tap root

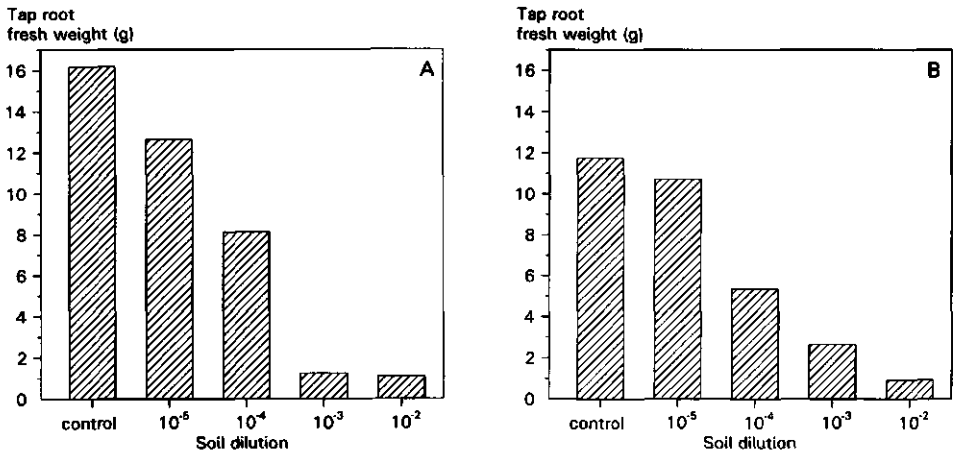

Fig. 2. Fresh weights (g, back-transformed from $\log_{10}$ values) of tap roots of bait plants grown for 18 weeks at five levels of infestation with BNYVV. See text for significancies. A. Expt 2a; B. Expt 2b.

weights for dilutions 10^{-4} to 10^{-2} (tap root weights relative to the control 46, 23 and 8%, $P < 0.01$), but not for dilution 10^{-5}.

Expt 3. Quantitative assessment of BNYVV and P. betae in soil after 3, 6 and 12 weeks

More plants were infected (Fig. 3A) and a 16-fold higher MPN of BNYVV ($P < 0.01$) was estimated after 6 weeks of incubation than after 3 weeks (Table 4). Differences between the MPNs of BNYVV after 6 and 18 weeks were not significant ($P = 0.05$), using either tap root or lateral root analysis (Table 4).

Numbers of *P. betae*-infected plants are presented in Fig. 3B. MPNs for *P. betae* were

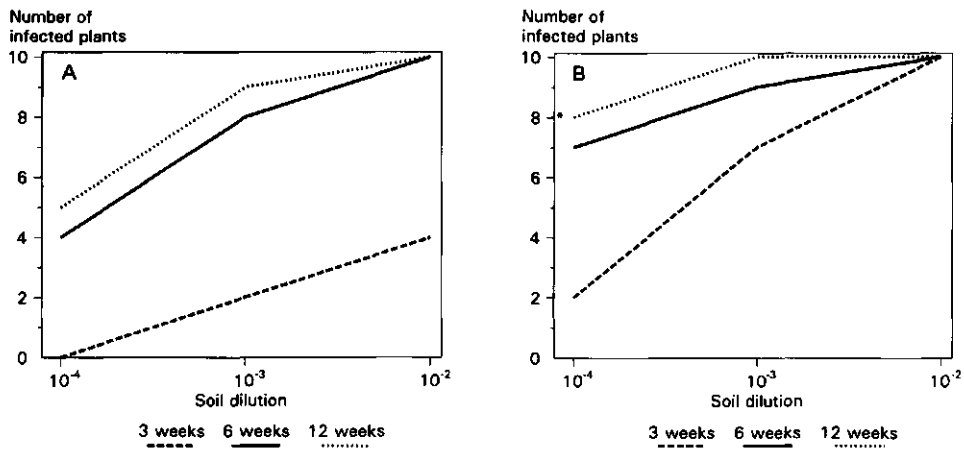

Fig. 3. Detection of BNYVV and *Polymyxa betae* in a serial dilution series of infested soil with sand, determined after 3, 6 or 12 weeks duration of the bioassay (Expt 3). A. BNYVV; B. *P. betae*.

Table 4. The inoculum potential of BNYVV and *Polymyxa betae* in soil as estimated by bioassay with 3, 6 or 12 weeks duration (Expt 3).

Duration (weeks)	Root parts analyzed for BNYVV	Most probable numbers of infective units ml^{-1} soil			
		BNYVV		*P. betae*	
		MPN	(95% confidence limits)[a]	MPN	(95% confidence limits)[a]
3	all	0.7	(0.3, 1.6)	6.8	(2.9, 16)
6	all	11	(4.8, 26)	22	(9.8, 53)
12	laterals	17	(7.4, 40)	81[b]	(35, 187)
12	tap root	8.6	(3.7, 20)		

[a] Confidence limits were calculated according to Cochran (1950).
[b] All plants of the three soil dilutions ussed were infected with *P. betae*. Two plants of the lowest dilution had white roots and might have been contaminated in a late stage, as also one of the non-infested control plants showed traces of *P. betae* at this time. The MPN is estimated with these two plants considered as being non-infected.

higher than for BNYVV (Table 4). A 6-week duration of the bioassay yielded a threefold higher MPN than a 3-week one ($P < 0.05$). With a 12-week duration, the MPN of *P. betae* had increased again ($P < 0.05$). The occurrence of traces of infection by *P. betae* in one control plant (with still white and healthy looking roots) at this time made the assessment less reliable. At the lowest dilution two suspected contaminated plants were taken into account (Table 4), however, more contaminations may have occurred.

Root development and symptoms of bait plants after 3, 6 and 12 weeks

Roots. Roots of bait plants developed well in the square pots. Volumes of tap and lateral roots are presented in Fig. 4. After 3 weeks, the whole soil volume was explored by the roots. At this time, total root weight and volume (log_{10}-transformed) were linearly affected by inoculum level ($P < 0.001$ and $P < 0.01$, respectively). Volumes in dilutions 10^{-2} and 10^{-3} were less than those in the control treatment ($P < 0.05$).

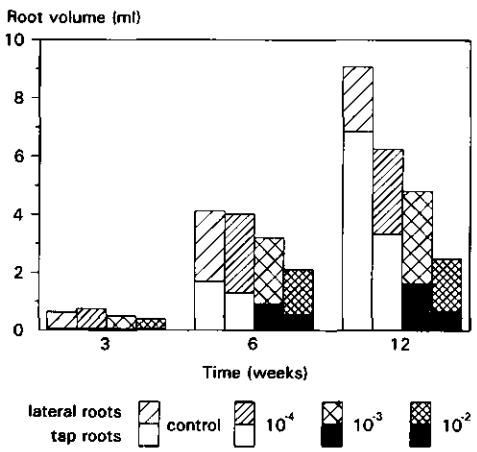

Fig. 4. Volumes of tap and lateral roots (ml, back-transformed from log_{10} values) of bait plants grown for 3, 6 or 12 weeks at four dilutions of BNYVV-infested soil (Expt 3). See text for significancies.

When bait plants had grown for 6 weeks, total root fresh weight (or volume, since volumetric weight was c. 1.0 g ml^{-1}) was linearly affected by inoculum level ($P < 0.001$). Total root weights at dilutions 10^{-2} and 10^{-3} were lower than the control ($P < 0.01$). Tap root volume in all four dilutions was less than in the control treatment ($P < 0.05$). Lateral root volume was significantly reduced at dilution 10^{-2}.

After 12 weeks, total and tap root weight showed a linear decrease with increasing inoculum levels ($P < 0.001$ for both parameters). Values for all three dilutions differed from those for the control ($P < 0.05$). Lateral root weights for none of the dilutions differed significantly from the control.

The rate of increase in fresh weight of roots from 3 to 6 weeks ranged from 0.18 g day^{-1} for the control, to 0.17, 0.14 and 0.09 g day^{-1} for the increasing levels of inoculum. During the next 6 weeks, the rates of increase in root weight were considerably lower at all four dilutions. In this period, the mean rates were 0.05 (42% of the control), 0.04 and 0.008 g day^{-1} for dilutions 10^{-4} 10^{-3} and 10^{-2}, respectively.

In a separate experiment the effects of addition of tiny amounts of pathogen-free soil to the sand medium on root growth was assessed. Addition of 1% of sterilized soil or less to coarse sand did not affect root weight.

Root diameters were measured for 80 randomly sampled lateral root pieces per dilution series per sample. The mean diameter decreased with increasing amount of infested soil in the sand medium ($P < 0.001$, at the three sampling times). Addition of increasing amounts of sterilized soil to sand resulted also, but to a lesser extent, in smaller diameters ($P < 0.05$) after a 3-week incubation period.

Symptoms. After a 3-week incubation period, leaf symptoms had not appeared and, occasionally, a discoloration of the roots was observed. After 6 weeks, numbers of BNYVV-positive plants had increased at all dilutions. Not all of these plants showed leaf symptoms, although the association was significant ($P < 0.001$). Dark-brown roots always contained BNYVV, light-brown roots always contained *P. betae*. In only 1 of the 15 plants with white roots, both vector and virus were detected. When bait plants had grown for 12 weeks, all but one (96%) of the plants with BNYVV-positive lateral roots showed leaf symptoms. All plants with BNYVV-containing tap roots showed leaf symptoms, but in tap roots of two plants with leaf symptoms the virus was not detected .

Discussion

Recovery of resting spores of P. betae by bioassay. Based on MPNs, the average recovery of *P. betae* from artificially infested sand was 1.7% of the resting spore clusters added. The procedure of sampling from the artificially infested sand lot followed by serial dilution did not introduce a large bias in MPNs estimated (a and b per series) in these experiments. Treatments with two different suspensions showed a slight difference in recovery: 2.2% for A versus 1.2% for B. In both suspensions, resting spore clusters had a similar mean size and size distribution, but they differed in virus content because they originated from a susceptible or resistant cultivar. More suspensions of each origin should be tested before the difference can be ascribed to the difference in cultivar. The effect of the origin of the spores on their virus content and on their recovery will be dealt with in a next publication (Tuitert et al., submitted).

A mean level of recovery between 1 and 2% implies that one infective unit of *P. betae* in the MPN assay represented 50–100 resting spore clusters in soil. Several reasons can be suggested for the low level of recovery. First, perhaps only a small proportion of newly formed resting spore clusters germinated and led to infection. Germination might depend

on the age of the resting spores, it is not known if endogenous dormancy is involved. Germination might also be influenced by dry storage after addition of the spores to the sand, although the negative effects of drying treatments of resting spores on numbers of bait plants infected were transient only (Tuitert, 1993). Second, the low level of recovery might imply that multiple infection of the rootlets is required to attain a detectable level of infection. Third, very likely, not all zoospores released from resting spores will succeed in causing infection; the efficiency of infection by zoospores is not known.

Keskin et al. (1962) observed that inoculating single resting spore clusters (containing 10–30 spores) to rootlets of seedlings resulted in infection in only 1–2% of the attempts. The low recovery is further in agreement with the results of Abe and Tamada (1986), who found that at least 50 resting spore clusters (each consisting of 35 spores) had to be applied to the roots of bait plants to obtain infection. In experiments by Fujisawa and Sugimoto (1976), infection was detected when plants were inoculated with 100–150 clusters and not with less than 50 clusters. The resting spores used in our experiments were obtained from 8-week-old plants. However, a similar level of recovery (1.5–1.9%) was found in preliminary experiments with resting spores from plants (cv. Regina) that had grown in *P. betae*- and BNYVV-infested soil for 6 months.

Lower limit of detection of BNYVV in soil. Prolongation of the 6-week duration of the bioassay on BNYVV to 18 weeks did not result in a lower dilution endpoint (Expt 2). The dilution endpoint in the assays was 10^{-4} or 10^{-5}. Büttner and Bürcky (1990) found a dilution endpoint of 2×10^{-3} for a heavily infested soil. However, their bioassay lasted only two weeks, but incubation temperature was higher. A more accurate estimation of the lower limit of detection could be made when spore clusters are added in fixed numbers to each individual plant. Expt 3 showed that analyzing the lateral roots (after 12 weeks) yielded more BNYVV-positives than analyzing the tap roots. Thus, the number of positives in Expt 2 might have been higher, both at 6 and 18 weeks, when lateral roots had been included in the analysis. In the standard application of the bioassay with a 6-week duration, root sap is obtained from the complete root system (Tuitert, 1990).

Quantitative estimation of BNYVV and P. betae in soil. In Expt 2, prolongation of duration of the bioassay from 6 to 18 weeks did not result in a significant higher MPN of BNYVV, neither did replanting of the pots after 6 weeks (Expt 2a). The results of Expt 3 confirmed the finding that extending the duration of the assay after 6 weeks did not result in a different estimate of the MPN of BNYVV. Apparently, roots had explored the pot volume early enough to enable primary BNYVV-infection to multiply beyond the detection limit of ELISA within 6 weeks for most of the plants. Under the prevailing environmental conditions, a 3-week bioassay appeared to be too short to enable either germination and infection of all viruliferous infective units, or sufficient multiplication of BNYVV after infection, or both. The MPN of BNYVV increased 16-fold from 3 to 6 weeks, the MPN of *P. betae* only 3-fold, showing that both aspects played a role, but BNYVV-multiplication in the plant apparently the major role.

After the three-fold increase in MPN from 3 to 6 weeks, the MPN of *P. betae* apparently still increased when incubation time was prolonged after 6 weeks (Expt 3). In view of the contamination detected after 12 weeks – traces of infection in a healthy looking control plant with white roots – the data for *P. betae* should be interpreted cautiously.

In conclusion, for comparison of levels of BNYVV-infestation in soil samples determination of the MPN after 6 weeks is satisfactory. Then the roots have adequately explored the pot volume and they can easily be handled with a handpress to obtain sap (Tuitert, 1990).

Symptom development in bait plants. After 6 weeks and more clearly after longer periods, a light brown colour of the roots was significantly associated with the presence of *P. betae*. Virus-free *P. betae* is known to cause a brownish discoloration (Tamada et al., 1990). Dark-brown discoloration combined with a high fragility of roots indicated BNYVV infection. Leaf symptoms were observed after 6 weeks, but were more pronounced in almost every BNYVV-positive bait plant after 12 or 18 weeks. After 18 weeks, three bait plants (out of 24 BNYVV-infected ones) showed typical systemic symptoms.

When pots of the 6-week bioassay were replanted once or twice, bait plants became highly infected, probably by the increased number of viruliferous *P. betae* spores present in the roots of the bait plants of the previous planting. The increased number of infective viruliferous units might have caused an earlier and more abundant primary infection of bait plants, resulting in an increasing virus content of bait plants of the second and third baiting.

Root weight of bait plants at different inoculum levels of BNYVV. In all experiments, total and tap root weight showed a progressive decrease with increasing levels of infestation. In the field, the negative relationship between (quantified) inoculum levels and root weight was also found (Tuitert and Hofmeester, in press). At a field level of infestation comparable to dilution 10^{-5} in the described experiments, root weight was not affected, but sugar content was lower than in non-infested soil (Tuitert and Hofmeester, in press).

BNYVV is considered to be the main cause of the effects on root growth observed in these experiments. *P. betae* itself might have contributed to, but unlikely be solely responsible for, the effects. A treatment with non-viruliferous *P. betae* was not included. Tamada et al. (1990) did not find any effect on root weight by virus-free *P. betae* when plants were grown for 40 days in a climate room followed by 3 months in a greenhouse. On the other hand, Blunt et al. (1991) reported that *P. betae*, which was assumed to be virus-free, reduced dry weight of (tap) roots of young plants. However, in their experiments, root weight was less reduced than that with viruliferous *P. betae* in our experiments. In view of their data, it may not be excluded that *P. betae* itself might have contributed to a decrease of root weight in our experiments. Isolates of *P. betae* might differ in aggressiveness. Gerik and Duffus (1988) found that three out of six isolates of *P. betae* reduced (lateral) root weight compared to that in non-infested soil in a 2-months assay, whether or not the isolates were viruliferous. Evidence for the greater role of BNYVV than of *P. betae* in causing root yield reduction in the field is derived from the results of a field trial with different initial inoculum levels of BNYVV (Tuitert and Hofmeester, 1992 and in press).

The infested soil used did not harbour other pathogens of sugar. Occasionally, resting spores of *Olpidium* were observed. The soil was not tested for the presence of BSBV (beet soil-borne virus). Differences in substrate because of making dilutions of the soil are probably of minor importance.

Proliferation of lateral roots (Expt 3). Effects of infection on volume of lateral rootlets were not as pronounced as those on the tap root. After 6 weeks, lateral root volume was reduced, but only at the highest inoculum level. After 12 weeks, effects depended on the inoculum levels. The root volumes of the two intermediate dilutions appeared to be higher and at 10^{-2} to be lower than that of the control. An initial reduction of lateral root weight of infected plants, followed by an increase later on, was found before (Tuitert et al., submitted). As BNYVV is known to cause both root necrosis and a proliferation of rootlets (Tamada et al., 1990), the net result of these effects will depend on the level of infection with the virus, and probably the vector.

In an early stage, differences in soil composition (soil:sand ratio) might have influenced the distribution of root diameters. Of the two infective agents, BNYVV will have been responsible for most of the reduction of root diameters. According to Gerik and Duffus (1988), BNYVV causes root tip death and increases root branching. Increased root branching, leading to proliferous extension of lateral roots, will result in a reduction of mean root diameter. The effect of BNYVV on root length will therefore not be proportional to the effect of the virus on root volume.

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Chapter 4

Effect of conditions during storage of infested soil on infection of bait plants by *Polymyxa betae* and beet necrotic yellow vein virus

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Effect of conditions during storage of infested soil on infection of bait plants by Polymyxa betae and beet necrotic yellow vein virus

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Abstract

Infectivity of resting spores of *Polymyxa betae* in soil stored air-dry or moist was determined by assessing infection of bait plants that were exposed to the soil. Storage of soil under air-dry conditions at room temperature resulted in a delayed onset of germination of resting spores compared to germination in soil stored under moist and cool conditions, as inferred from the infection of the bait plants. Bait plants had to be exposed for more than 12 h to flooded infested soil before germination and infection had occurred. However, when soil was prewetted for 24 h before exposing bait plants, germination, infection and transmission of beet necrotic yellow vein virus (BNYVV) were accomplished within 12 h, but only with the moistly stored soil. When resting spores isolated from roots were stored for 4 and 8 weeks under dry conditions at 22 °C, germination of viruliferous spores, as measured by detection of BNYVV in bait plants exposed for 48 h to the spores, was less than that of spores stored in moist soil at 22 °C. Approximately 100% of bait plants were infected after exposure to resting spores that were frozen in demineralized water or stored cool (5 °C) in water or moist soil for 42 weeks. Air-dry cool storage for 42 weeks resulted in a low percentage of infection. Storage conditions of soil influence the results of bioassays for detection of rhizomania when short baiting periods are applied, whereas differences in infectivity were not detected using a bioassay with long duration.

Additional keywords: BNYVV, sugar beet, bioassay, germination, dormancy, resting spores.

Introduction

Polymyxa betae Keskin (Plasmodiophoromycetes) is the soil-borne fungal vector of beet necrotic yellow vein virus (BNYVV), the causal agent of rhizomania disease of sugar beet. Resting spores of this fungus can survive in moist or dry soil for at least 15 years and still transmit the virus (Abe and Tamada, 1986). Resting spores germinate in the presence of host roots, releasing primary zoospores that infect epidermal root cells (Keskin, 1964). Storage conditions of soil can affect germination of fungal resting spores (Sussman, 1976). At two institutes participating in a co-operative trial (Tuitert and Hofmeester, 1992), soil samples were stored under different conditions, air-dry at 20 °C and moist at 5 °C, prior to assessment of BNYVV and *P. betae* by bioassay. Consequently it was considered important to determine what effect these storage conditions had on the resting spores of *P. betae*. The effect on quantitative assessment of *P. betae* and BNYVV using a 6-week bioassay was reported previously (Tuitert, 1990). In this paper, effects of different storage conditions of soil or of isolated resting spores on the onset and rate of germination of the resting spores of *P. betae*, as inferred from infection of bait plants, were investigated.

Materials and methods

Effect of storage conditions of naturally infested soil on infectivity of resting spores of P. betae

BNYVV-infested soil (sandy clay, pH-KCl 7.5, organic matter 5%) was collected in spring from a field in Tholen, the Netherlands, where sugar beet had been grown in the three preceding years. The moist soil was sieved (5 mm), mixed and stored under different conditions:

MC = moist (pF 2.2–2.5, gravimetric water content c. 16%) and cool (5 °C);
DW = air-dry at room temperature (c. 20 °C).

The effect of these storage conditions on germination of resting spores in the soil was determined indirectly by assessing infection of bait plants exposed to the soil. The transmission of BNYVV by zoospores released from the resting spores was determined by ELISA of the exposed bait plants. The detection procedure was adapted from bioassay methods described by Westerlund et al. (1978) for *Olpidium brassicae* and Beemster and De Heij (1987) for *P. betae*. Samples of 5 g of dry soil or an amount of moist soil corresponding to 5 g of dry soil were placed in Petri dishes (diameter 14.5 cm), mixed with 45 g of sterilized coarse sand and wetted by adding 60 ml of demineralized water. Sixteen 2–3 week-old sugar beet seedlings cv. Regina, were used as bait plants in the Petri dishes with their roots spread out over the soil surface. The dishes were partly covered with an opaque lid to reduce evaporation and to keep the soil slurry in darkness. Water was supplied when necessary to compensate for evaporation. The dishes were placed in a growth chamber at a constant temperature of 22 °C with 16 h light. Bait plants were removed from the soil after 12, 24, 48 or 72 h of exposure. Their roots were carefully washed in running tap water and 12 of the 16 bait plants were planted separately into individual pots containing 200 ml of sterile coarse sand. The plants were placed, randomized, in the greenhouse with day (16 h) and night temperatures of c. 23 and 15 °C, respectively. After 6 weeks, the plants were checked for the presence of *P. betae* by light microscopy and of BNYVV by ELISA (Tuitert, 1990). Controls consisted of Petri dishes containing 50 g of sterilized coarse sand, on which bait plants were exposed for periods identical to the infested series. Experiments 1 to 4 were performed consecutively after storage periods of the infested soil of 12, 24, 30 and 40 months, respectively. Modifications to this general procedure for each Experiment (1–4) are given separately.

Expt 1. A comparison of two inoculum densities. In a preliminary experiment two amounts of infested soil were tested, either 5 g or 0.5 g, both made up to 50 g with sterile sand. The exposure period of bait plants in the Petri dishes was 48 h, with alternating temperatures of 22 and 15 °C (16/8 h, respectively). Treatments *MC*, *DW* and one extra treatment (*MCa*), where moist soil was air-dried for 1 day prior to baiting, were tested. One Petri dish per treatment was used for exposing bait plants to the soil-borne inoculum.

Expt 2. A comparison of exposure periods and the effect of two drying treatments. Three different periods of exposure of bait plants to infested soil (5 g) were compared. Bait plants were incubated in the Petri dishes for 24, 48 or 72 h. Beside the treatments *MC* and *DW*, two extra treatments, *MCa* and *MCb*, were included. For treatments *MCa* and *MCb*, moist soil was air-dried for 1 and 7 days, respectively, prior to baiting. One Petri dish per treatment was used for exposing bait plants to the soil-borne inoculum.

Expt 3. A comparison of three exposure periods. Exposure periods of 12, 24 and 48 h were compared for treatments *MC* and *DW*. All treatments were performed in duplicate:

two Petri dishes per treatment were used for exposure of bait plants.

Expt 4. A comparison of four exposure periods and the effect of prewetting. Expt 3 was repeated with the addition of a 72-hour exposure period. Also, a prewetting treatment was included where 60 ml water was added to the soil 24 h before bait plants were applied (*MC'* and *DW'*). The latter treatment was only investigated with a 12-hour exposure period of bait plants to the soil. Every treatment was performed in duplicate, as described for Expt 3.

Effect of storage conditions on infectivity of resting spores isolated from roots
A suspension of resting spore clusters was prepared from lateral roots of 5-month-old sugar beet plants, cv. Regina, which had been grown in a mixture of 15% (v/v) BNYVV-infested soil with coarse sand. The roots were cut up, macerated for 2 min in a Waring blender and ground with a pestle and mortar. After addition of water, the suspension was sieved through Monodur gauze (50 μm) by vacuum filtration to remove large root debris. This root debris was once more ground in the mortar, resuspended and filtered. In order to increase the density of resting spores, the filtrate was centrifuged (at approximately 2000 *g* for 3 min) and the pellet was resuspended in demineralized water. Five ml of the suspension, containing approximately 10^6 resting spore clusters, was pipetted gradually onto cellulose-nitrate microfilters (Sartorius, pore size 3 μm and 5 cm filter diameter), while the water was removed by vacuum filtration. Zoosporangia were not observed in the filtrate. Filters with spores were left to air-dry. A thin layer of Glisseal laboratory grease was applied along the margins of the filters and a spore-free filter was stuck on top, to create resting spore 'sandwiches'. For the non-infested controls, water was applied to the filters in stead of the suspension. The 'sandwiches' were stored under the following conditions:

1. in a mixture (50/50) of non-infested moist field soil and coarse sterile sand (pH-KCl 7.7, gravimetric water content 13%);
2. in a similar mixture, but with autoclaved field soil;
3. in demineralized water;
4. dry in the air.

All samples were stored in the dark, with alternating temperatures of 22 and 15 °C (16/8 h, respectively), except for two sandwiches which were tested immediately after preparation.

After 4 and 8 weeks storage, two sandwiches per treatment were taken for assessment of germination of viruliferous resting spores by indirect bioassay. The sticky margin of the sandwiches was cut off and both filters of each sandwich were placed on the bottom of a Petri dish (diameter 8.5 cm) with 25 ml of diluted Steiner nutrient solution (10%). Twenty 2–3 week-old sugar beet seedlings cv. Regina were placed in each dish, with their roots covering the filters. The dishes were partly covered and placed for 48 h in a growth chamber with alternating temperatures of 22 and 15 °C (16/8 h, respectively). Plants were removed from the dishes, their roots were washed with running tap water and they were individually planted in pots containing 200 ml of sterile sand. These pots were placed under greenhouse conditions as described before. After 6 weeks, roots of the bait plants were tested for the presence of BNYVV. BNYVV was the indicator of infection by viruliferous *P. betae*.

To investigate the effects of a long storage period, two filters per treatment were assayed after storage at 5 °C for 42 weeks. In addition, two filters from treatment 3 were kept frozen at –18 °C for 42 weeks.

Statistical analyses

The frequency tables of infected plants from the different treatments and exposure periods were analyzed by generalized linear modelling with a logistic link function and a binomial error distribution, using GENSTAT 5 (Payne et al., 1988). The deviances of factors were compared with χ^2-values to determine their significances. In Expt 1, the effect of increasing the period of drying the soil before the assay on the number of plants infected by *P. betae* or BNYVV was tested for each of the two amounts of soil separately. In Experiments 3 and 4, the main effects analyzed were storage conditions (two) and exposure period (three and four, for Expt 3 and 4, respectively). For the 12-hour exposure period in Expt 4, the effect of prewetting the soil on numbers of infected plants was tested separately.

Results

Experiments with naturally infested soil. Expt 1 showed that 5 g was an adequate amount of soil to use in this type of experiment (Table 1). Exposure to dry soil, air-dried for 1 day (*MCa*) or stored dry for 12 months (*DW*), yielded lower numbers of infected plants (*P. betae* and BNYVV) than exposure to the moist (*MC*) soil. In Expt 2, with an additional air-drying treatment, a decreasing number of infected plants with increasing time of drying of soil prior to exposure was found, but only for the 24-hour exposure period (Table 2). At longer periods of exposure, the number of bait plants was not adequate to detect differences in infection between treatments.

In Experiments 3 and 4, significantly higher numbers of bait plants were infected when exposed to *MC* than to air-dried (*DW*) soil. The numbers of *P. betae*- and BNYVV-infected plants increased significantly with increasing exposure period (Tables 3 and 4). Exposure of roots for 12 h to either *MC* or *DW* soil was too short to detect any infection of bait plants.

Not only was the time of onset of germination of spores delayed when infested soil had been stored dry and warm, also the rate of increase of germination (inferred from the rate of progress of infection) appeared to be slowed down. For *MC* soil, a 79% increase of

Table 1. Effect of storage conditions of infested soil on germination of resting spores of *Polymyxa betae* and transmission of BNYVV, as measured by the infection of bait plants exposed for 48 h to two quantities of infested soil (Expt 1).

Storage conditions [a]		Numbers of infected bait plants ($N = 12$)			
		P. betae		BNYVV	
		5 g soil	0.5 g soil	5 g soil	0.5 g soil
MC	Moist, 5 °C	12	6	6	2
MCa	Moist, 5 °C air-dried 1 day	6	4	1	0
DW	Air-dry, 20 °C	0	1	0	0
Significance [b]		$P < 0.001$	$P < 0.10$	$P < 0.001$	$P < 0.10$

[a] Duration of storage of the soil was 12 months.

[b] Significance of the decrease in numbers of infected plants from treatment *MC* to *MCa* to *DW*, per quantity of soil.

Table 2. Effect of storage conditions and air-drying treatments of infested soil on germination of resting spores of *Polymyxa betae* and transmission of BNYVV, as measured by the infection of bait plants exposed for 24, 48 or 72 h to the wetted infested soil (Expt 2).

Storage conditions [a]		Numbers of infected bait plants ($N = 12$)					
		P. betae			BNYVV		
		24 h	48 h	72 h	24 h	48 h	72 h
MC	Moist, 5 °C	12	12	12	6	12	11
MCa	Moist, 5 °C air-dried 1 day	5	11	12	4	11	11
MCb	Moist, 5 °C air-dried 7 days	2	12	12	1	12	12
DW	Air-dry, 20 °C	0	12	12	0	9	12
Significance [b]		$P < 0.001$	n.s.	n.s.	$P < 0.01$	n.s.	n.s.

[a] Duration of storage of the soil was 24 months.
[b] Significance of the decrease in numbers of infected bait plants with increasing time of drying of soil prior to baiting (from *MC* to *DW*).
n.s. = not significant.

Table 3. Effect of moist/cool and dry/warm storage of infested soil on germination of resting spores of *Polymyxa betae* and transmission of BNYVV, as measured by the infection of bait plants exposed for 12, 24 or 48 h to the wetted infested soil (Expt 3).

Storage conditions[a]		Mean numbers of infected plants ($N = 2 \times 12$)[b]					
		P. betae			BNYVV		
		12 h	24 h	48 h	12 h	24 h	48 h
MC	Moist, 5 oC	0	11	12	0	8.5	10.5
DW	Air-dry, 20 oC	0	0	8.5	0	0	3

[a] Duration of storage of the soil was 30 months.
[b] Each treatment consisted of an assessment of two lots of 12 plants. The main effects, storage condition and exposure period, were analyzed separately for *P. betae* and BNYVV. The two storage conditions resulted in different numbers of infected plants, $P < 0.001$. Numbers of infected plants increased with increasing period of exposure ($P < 0.01$), for both *P. betae* and BNYVV. Interaction of the two treatments was not significant.

infection by *P. betae* (from 0 to 9.5 out of 12 test plants) required less than 12 h, for *DW* soil a 38% increase (from 4.5 to 9 out of 12 test plants) occurred in 24 h (Table 4). This apparent effect on the rate of increase was not detected as a statistically significant interaction between storage treatment and exposure period.

Pre-wetting of infested soil for 24 h before exposure of bait plants caused an increase in infection with *MC* soil, but not with *DW* soil at the 12-hour exposure period (Table 4).

After air-drying the baited soil samples of Expt 4, to kill zoospores released by the first baiting, all were baited a second time, with a 72-hour exposure period. On every soil sample, infection of bait plants occurred during the second baiting.

Table 4. Effect of moist/cool and dry/warm storage of infested soil on germination of resting spores of *Polymyxa betae* and transmission of BNYVV, as measured by the infection of bait plants exposed for 12, 24, 48 or 72 h to the wetted infested soil. The effect of prewetting of soil for 24 h before baiting was tested with a 12-hour exposure period (Expt 4).

Storage conditions[a]	Mean numbers of infected bait plants ($N = 2 \times 12$)[b]							
	P. betae				BNYVV			
	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h
MC Moist, 5 °C	0	9.5	11.5	11.5	0	7	9.5	11
DW Air-dry, 20°C	0	0.5	4.5	9	0	0	1	2
MC' Moist + prewetted	8.5				7			
DW' Dry + prewetted	0				0			

[a] Duration of storage of the soil was 40 months.

[b] Each treatment consisted of an assessment of two lots of 12 plants. Effects of storage condition and exposure period were analyzed for *P. betae* and BNYVV separately. The difference in numbers of infected plants between the two storage conditions of soil was significant at $P < 0.001$. Numbers of infected plants increased with increasing exposure period ($P < 0.001$). Interaction of the two treatments was not significant. For the 12-hour exposure period, the effect of prewetting was analyzed separately: prewetting of moist soil resulted in an increase in the numbers of infected plants ($P < 0.001$) compared to moist soil that was not prewetted.

Experiments 2, 3 and 4 were performed at the same temperature (22 °C) during exposure of the bait plants. These experiments gave corresponding numbers of infected plants for similar treatments and exposure times, indicating that the duration of storage of soil had no effect on the behaviour of the resting spores and/or the released primary zoospores, at least between 24 and 40 months of storage.

Table 5. Effect of various storage conditions for periods of 4, 8 or 42 weeks on germination of viruliferous resting spores of *Polymyxa betae* isolated from roots, as measured by BNYVV infection of bait plants. Bait plants were exposed to wetted resting spores on microfilters for 48 h.

Storage conditions[a]	Mean numbers of BNYVV-infected bait plants ($N = 2 \times 20$)[b]			
	4 weeks, 22 °C	8 weeks, 22 °C	42 weeks, 5 °C	42 weeks, −18 °C
Moist soil	15	19.5	19	n.t.
Moist soil, autoclaved	17.5	20	20	n.t
Water	3	10.5	20	18.5
Air (dry)	0	9	3	n.t.

[a] Temperatures during storage for 4 and 8 weeks were 22/15 °C (16/8 h) and during 42 weeks constant 5 °C or −18 °C.

[b] Each treatment consisted of an assessment of two lots of 20 plants. Analysis of the data for storage periods of 4 and 8 weeks showed that the number of infected plants was significantly affected by storage conditions ($P < 0.001$), period of storage ($P < 0.001$) and the interaction of these factors ($P < 0.05$).

n.t. = not tested.

Experiment with resting spores isolated from roots. The fresh resting spores on filters were able to cause infection; an average number of 15.5 out of 20 bait plants became infected when spore sandwiches were baited directly after preparation. Storage of resting spores either in water or air-dry, resulted in less infection of bait plants than with storage in soil, either non-sterile or autoclaved (Table 5). With an increase in storage time from 4 to 8 weeks, infection of bait plants increased, but this increase was dependent upon the storage conditions (interaction between storage treatment and period).

When the spore 'sandwiches' were stored cool for 42 weeks, infection percentages were approximately 100% for all moist treatments, either soil or water. The spores that were stored under dry conditions gave rise to low percentage infection. The level of infection resulting from frozen spores was the same as that from spores stored in water at 5 °C.

Discussion

Onset and rate of germination of resting spores in naturally infested soil. Irrespective of the preceding storage conditions of soil, bait plants had to be exposed for more than 12 h to flooded naturally infested soil in order to become infected, when no specific pre-treatments were applied. When bait plants were exposed to infested soil for 24 or 48 h, higher numbers of *P. betae*- and BNYVV-infected plants were detected for soil stored moist and cool than for soil stored air-dry at room temperature. With *DW* soil, the onset of germination was delayed; infected plants were rarely detected after 24 h exposure. Prewetting the moistly stored soil showed that germination of and infection by *P. betae*, with transmission of BNYVV, can take place within 12 h.

Effects of different treatments on germination of resting spores in soil is difficult to study on the germination process itself. The effects of storage conditions of soil on the incidence of infected bait plants was probably mainly due to an effect on the resting spores, rather than on the zoospores after they are released from the resting spores. Consequently, treatment effects on germination of resting spores are inferred from the indirect assessment, i.e. bait plant infection. Dry or moist storage conditions were at different storage temperatures. However, in the experiment with isolated resting spores, four storage treatments were compared at the same temperature. As under these conditions a dry treatment resulted in a lower infectivity than moist soil treatments (Table 5), moisture during storage is assumed to be more important than temperature in determining infectivity or rate of germination of resting spores by bioassay, in Experiments 1–4.

The numbers of bait plants and exposure periods tested were adequate to detect differences in the onset of germination of resting spores between different treatments. However, differences could only be detected with short periods of exposure. A smaller amount of infested soil, a higher number of bait plants and a number of intermediate exposure times, in various combinations or individually, would have been necessary to allow an accurate determination of response curves and infection rates.

Exposure periods longer than 72 h were avoided, because of the risk of secondary infection of plants by zoospores released from neighbouring plants. The minimum time needed for resting spores to germinate in the presence of host roots has not yet been reported. However, the results in Table 4 indicate that, with a pre-treatment of wetting the soil, spores can germinate and lead to infection within 12 h. From initial zoospore infection to release of secondary zoospores takes approximately 70 h at 22 °C (Ivanović et al., 1983). Once secondary zoospores are released, they can cause new infections within 30 min (Peters and Godfrey-Veltman, 1989).

As for the time of onset of germination; Bouhot (1988) mentioned that penetration into root hairs occurred the second day after contact between roots and *P. betae*. He did not

state whether the inoculum was infested soil or resting spores isolated from roots. Experiments carried out by Slykhuis (1974) on WSSMV transmission by *Polymyxa graminis*, showed that after exposure for 1 day to infested soil stored under dry conditions, none of the bait plants were infected, and after exposure for 2 days only 1% of bait plants were infected.

Observations made with the baiting procedure are not independent, as has been discussed earlier (Tuitert, 1990). In theory, zoospores from one resting spore cluster might be able to infect more than one bait plant. However, as the number of bait plants was not very high, resulting in a low density of roots, the chance that zoospores from one resting spore cluster infected more than one bait plant will have been small.

The results for BNYVV reflect those for *P. betae*. Transmission of BNYVV by viruliferous spores in soil can be accomplished within 12 h. There is no information on the rate of germination of viruliferous versus non-viruliferous resting spores. The present study does not allow to draw conclusions on this aspect either.

All soil samples from Expt 4 gave rise to infection when baited for a second time. From the point of view of survival strategy, it is advantageous that not all resting spores had germinated within 72 h.

Effects of drying and pre-treatment of soil on infectivity. Dry/warm storage conditions probably induced dormancy of the resting spores, whereby spores needed a longer period of hydration before they were able to germinate and also showed a greater variation in germination (resulting in a lower rate of infection) than when they were stored under moist/cool conditions. The longer the soil was dried, the more spores became dormant or the longer the period necessary for hydration; drying for only 1 or 7 days reduced the infectivity of the *MC* soil compared with direct assessment of this soil (Tables 1 and 2).

With another fungal virus vector, *Olpidium brassicae*, comparison of germination of resting spores in freshly collected and air-dried soil (Westerlund et al., 1978) yielded results similar to those for *P. betae*. With *O. brassicae*, the dormancy period after air-drying appeared to be longer. Six to eight days of exposure of bait plants to soil previously stored under dry conditions were needed for infection by *O. brassicae*, whereas exposure for 48 h to moist soil caused 100% infection. Somerville (1894) mentioned that soil infested with *Plasmodiophora brassicae* had to be kept in a moist condition because he had experienced that soil 'became useless for purposes of infection if it was allowed to become over-dry'.

Slykhuis (1975) studied the effect of drying of soil on the transmissibility of WSSMV by *P. graminis*. He observed an increase in infectivity in a 90–120 day bioassay after air-drying the soil samples for 4–5 months, as compared to the infectivity directly after collecting the soil. This increase should, however, not merely be ascribed to the drying of soil. Duration of storage might have been responsible for maturation of spores; storage under moist conditions might also have resulted in higher infection, but this was not tested.

Prewetting the *MC* soil for 24 h altered the germination of spores in such a way that a high incidence of infection by *P. betae* was observed after only 12 h exposure (Table 4). It is generally assumed that root exudates are required as a stimulus for infection (Habibi, 1969). Apparently, the continuous presence of plant roots is not required to induce a germinable state. This may be an example of Hawker and Madelin's (1976) statement that germination of spores often depends on two sequential signals, the first is a dormancy-breaking one (in this case hydration), priming the spore for receipt of the second specific signal that actually evokes germination. The results of Table 4 suggest that not only in *DW* soil, but also in the moistly stored soil (*MC*) some of the spores were dormant.

Twenty-four hour of prewetting was too short to detect any effect on the spores in the dry soil with a 12-hour exposure period. After a 4-day exposure of bait plants to soil infested with *O. brassicae* and stored under air-dry conditions, no plants became infected when prewetting of soil lasted 1 day, but all plants were infected when it lasted 6 days (Westerlund et al., 1978). Pre-treatments other than wetting were not tested, but heat treatment apparently also stimulates germination of *P. betae* (Beemster and De Heij, 1987). Various treatments can break constitutive or exogenous dormancy of fungal spores, e.g. flooding, alternate wetting and drying, prolonged drying, freezing and heat treatment (Sussman and Halvorson, 1966).

Infectivity of viruliferous resting spores isolated from beet roots. Exposure of bait plants for 48 h to microfilters covered with viruliferous resting spores of *P. betae* isolated from roots resulted in a high percentage infection with BNYVV. The amount of resting spores applied was high, to compensate for the finding that only a fraction of fresh clusters germinate (Tuitert et al., submitted), and that not all the infective clusters will be carrying BNYVV (Fujisawa and Sugimoto, 1977; Tuitert et al., submitted). The filters were dried before sealing them into a sandwich, this would have killed any zoospores that were present (Campbell and Lin, 1976). When dry conditions were prolonged for 4 weeks at 22/15 °C or spores were kept in demineralized water, the germination of viruliferous spores was reduced compared to germination of those that had been stored in moist soil (inferred from the infection of the bait plants, Table 5). The effect of dry conditions, both at 22/15 °C and at 5 °C, corresponded with the results of the experiments with naturally infested soil; apparently the hydration of dry spores requires time.

For BaYMV, transmitted by *P. graminis*, Usugi (1988) reported that the potency of dried roots or roots stored in flooded soil at 4 °C as a source of inoculum increased after they had been buried in moist soil at 23 °C for over 14 days. This finding is in line with the reduced infection from *P. betae* spores stored dry or in water for 4 weeks (Table 5).

It is known that soil extracts often stimulate fungus spores (Sussman and Halvorson, 1966). Nevertheless, it is difficult to speculate on the causes for the lower infection levels after storage of spores in water for 4 and 8 weeks than after storage in moist soil (pH, ion concentration, oxygen), because after 42 weeks storage differences between water and soil storage were not detected. Repetition of this experiment and further investigations into the germination process would be required to explain these findings.

Implications of the effects of storage conditions for detection procedures. When short baiting periods are used in methods for detection of BNYVV and *P. betae* in soil, storage conditions influence the results of assessments. This risk might be diminished when a pre-treatment of soil is applied (Beemster and De Heij, 1987). The effects of storage conditions on germination of *P. betae* and transmission of BNYVV should also be considered when infested soil is used as source of inoculum in screening for resistance.

Differences in infectivity between spores stored under different conditions were not detected when plants were exposed to the soil for long periods of time: with a 6-week bioassay on serial dilutions of *MC* and *DW* soil, estimates of the inoculum level in the soil were similar, for both *P. betae* and BNYVV (Tuitert, 1990). These equal estimates also demonstrated that in the baiting experiments a difference in decline of inoculum could be excluded as the cause of the difference observed in infectivity of the inoculum in the *MC* and *DW* soils during the 12–40 months storage period. The duration of storage of soil neither influenced the infectivity of the soil in short-lasting baiting experiments: Experiments 2, 3 and 4 showed corresponding numbers of infected plants for similar treatments and exposure times.

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Chapter 5

Epidemiology of beet necrotic yellow vein virus in sugar beet at different initial inoculum levels in the presence or absence of irrigation:

Disease incidence, yield and quality

dry soil. The relationship between the MPNs determined and root weight, sugar content and sugar yield at harvest could be described by Gompertz curves. The increase in disease incidence with increasing MPN in 1989 was adequately fitted with a logistic equation.

Introduction

A fundamental component of quantitative epidemiological investigations involving soil-borne pathogens is the relationship between inoculum density and disease incidence and severity. This paper presents the results of a study on this relationship for rhizomania disease of sugar beet, caused by beet necrotic yellow vein virus (BNYVV). Environmental factors, such as soil moisture and temperature, the susceptibility and sensitivity of the beet cultivar and the aggregation of inoculum can influence the inoculum density – disease relationship. As the fungal vector *Polymyxa betae* Keskin infects the roots by means of zoospores, moist conditions, e.g. through irrigation, will favour disease development. The inoculum density – disease relationship of this pathosystem has been studied under controlled conditions in pot experiments with dilutions of infested soil [Bürcky et al., 1986; Bürcky and Beiss, 1986]. It has not been studied under field conditions.

Therefore, a field experiment was set up in 1988 to study the development of disease at different inoculum levels of BNYVV. The influence of soil moisture conditions was investigated by applying drip irrigation. Inoculum levels were created by artificial infestation of a disease-free field with different amounts of infested soil.

In this paper, the effects of inoculum levels of BNYVV on disease incidence, sugar content, root weight and quality parameters of susceptible sugar beet in three successive years are reported. The quantification of inoculum build-up during two years was published previously [Tuitert and Hofmeester, 1992]. Part of the first-years' results were presented in preliminary reports [Hofmeester and Tuitert, 1989; Tuitert and Hofmeester, 1990].

Materials and methods

General description of the field trial

The field trial, for description and lay-out see Tuitert and Hofmeester [1992], was situated in the Noordoostpolder on a calcareous clay soil with a pH-KCl of 7.4 and 4.2% organic matter. The soil water retention curve is given in Fig. 1. The experiment was arranged in a split-plot design, with two irrigation levels as main plots, five inoculum level subplots and all in four blocks. Plot size was 6×10 m^2. Sugar beet cv. Regina was drilled in three consecutive years in 50 cm rows and a sowing distance of 18.5 cm

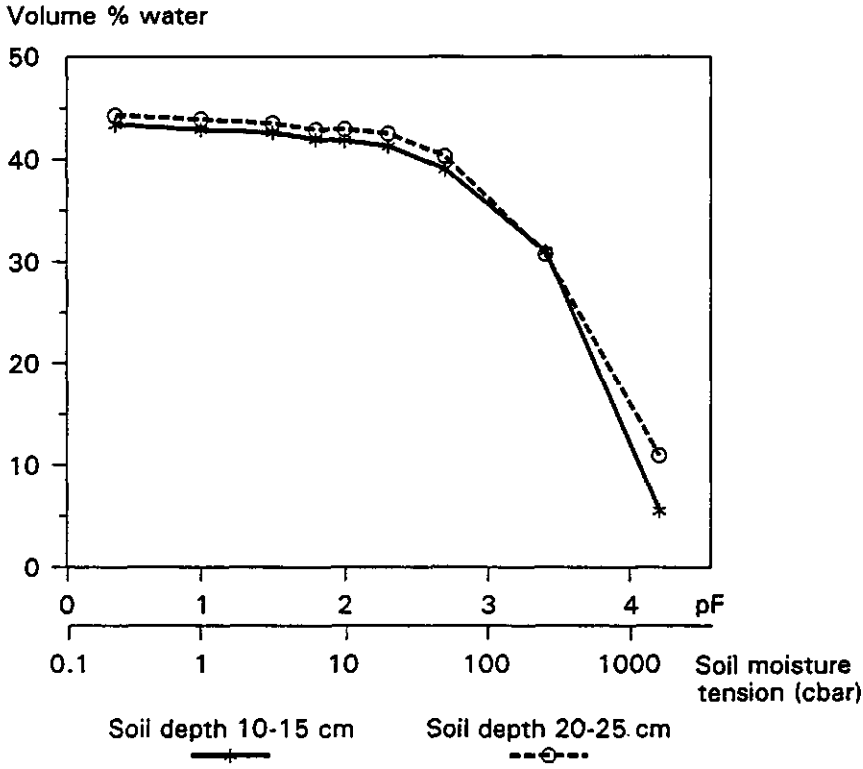

Fig. 1. Soil water retention curve for the trial field soil.

within the row. Sowing dates are given in Table 1. Irrigation was applied along every row with a drip irrigation system with emitters spaced 30 cm apart. Soil moisture tension was recorded by tensiometers three to five times a week at 15 cm and 30 cm depth. After dry periods, when soil moisture tension (soil water potential presented as a positive value; Hillel, 1982] at 15 cm depth exceeded *c.* 20 cbar (20 kPa), drip irrigation was used to supply around 10 mm water in 2 to 3 hours. The times of irrigation are indicated in Fig. 2. One of the four blocks was excluded from the analyses, because of the poor emergence and development of the plants in all three years caused by soil structure problems.

Fertilizer was applied prior to drilling at rates recommended on the basis of soil analysis. Pelleted seed was used, treated with thiram, hymexazole and furathiocarb. In the second and third year granular carbofuran was applied at drilling. Beet cyst nematodes were not detected in soil samples taken before the first, second and third beet crop. Generation of the inoculum levels, assessment of BNYVV in the soil by bioassay and calculation of most probable numbers (MPN 100 g^{-1} of soil) of infective

Table 1. Experimental details of the field trial in 1988, 1989 and 1990

	Year		
	1988	1989	1990
Sowing date	15 April	2 May	2 April
Harvest date	18 October	27 September	12 October
Length of growing season (days)	186	148	193
Date of 50% emergence	26 April	27 May	17 April
Periodical sampling date (weeks after sowing)	24 May (6)[a] 4 July (11) 23 August (19)	22 June (7)[a] 21 August (16)	18 June (11) 6 August (18)
Precipitation (> 1 mm)[b]			
– frequency (during growing season)	52	37	60
– amount (mm)	421	292	453

[a] Analysis of virus content only.
[b] Irrigation excluded.

units of BNYVV [Tuitert, 1990] were described before [Tuitert and Hofmeester, 1992].

Plant sampling and analysis

At harvest, all plants of the central 4×5 m^2 of each plot were taken for determination of root weight, sugar content, sodium and α-amino nitrogen contents of the root. Sugar content was determined by polarimetry [Anonymus, 1990], sodium by flame photometry and α-amino nitrogen by fluorimetry, all performed according to the standard procedures used in Dutch sugar industry [Anonymus, 1992]. The last two components, together with potassium, negatively influence the extractability of sugar and are referred to as (additional) quality parameters in this paper. Rhizomania affects the contents of Na, α-amino N and K in the root [Müller, 1983; Graf and Isak, 1986; Bürcky et al., 1986]. Potassium content is not presented because it is not a sensitive indicator of plant infection with BNYVV, as compared to the other parameters [Heijbroek, 1989]. Ten root tips were collected by 'every kth' systematic sampling [Cochran, 1953] as described for application in virus surveys [Barnett, 1986]. The number of roots with symptoms of rhizomania was determined. Where the diameter of the root tip was approximately 1 cm, a small piece (1–2 cm) was cut from which sap was collected by handpress. Assessment of BNYVV was by means of double antibody sandwich ELISA [Clark and Adams, 1977], as described before [Tuitert, 1990].

In two adjacent sampling area's of 4×1 m^2 each (at either side of the central harvest area) samples were taken periodically, according to a

pre-determined scheme. Of ten plants, root tips were analyzed by ELISA and the remaining plant parts used for dry matter and mineral content assessments [Haverkort et al., unpublished]. Twenty plants were used for determination of root weight, sugar content and additional quality parameters. The 20-plant weights were converted to weights per ha. Sampling dates in the three years are shown in Table 1.

Statistical analysis

Results at each sampling date were analyzed by analysis of variance (ANOVA) of a split-plot design, using GENSTAT 5 [Payne et al., 1988]. Disease incidence (percentage of infected plants) was angular transformed [Mead and Curnow, 1983] before analysis. ANOVA of disease incidence was performed including time as an experimental factor; the repeated-measures design within each year [Campbell and Madden, 1990] was taken into consideration. There was no need to consider temporal autocorrelation of the error [Madden and Campbell, 1990], because successive samplings within a year were destructive [Zadoks, 1978] and plants adjacent to empty positions from the previous sampling were not taken. The number of sampling times was not adequate for disease progress curve analysis within a year [Campbell, 1986]. Regression was applied for analysis of the relationship between inoculum potential ($\log_{10}$ MPN + 1) and yield parameters in 1989 and 1990. The results of linear and nonlinear regression (polynomial with a quadratic term, exponential, line + exponential, logistic and Gompertz curves) were evaluated by means of their significance (F-test), the percentage of variance accounted for (R^2 adjusted) and plots of standardized residuals versus fitted values. Because of the split-plot design, effects of irrigation were investigated by fitting curves for each strip separately, and comparing the parameters by ANOVA [Campbell and Madden, 1990]. However, the comparison of the parameters of the nonlinear models will still be approximate, as the X-values (log MPNs) of the non-irrigated and irrigated sets have different positions (on the X-axis) and therefore the different parameters are not all estimated with the same precision. This should be taken into consideration, especially for the 'extrapolated' asymptotes. The nonlinear relationship between disease incidence and inoculum potential ($\log_{10}$ MPN + 1) was investigated by fitting different disease progress models to the untransformed data (using the 'Fitnonlinear' directive of GENSTAT 5): the monomolecular or negative exponential model, the logistic and the Gompertz model were compared. For the logistic and Gompertz model, the lower and upper asymptote were set at 0 and 1 (limits of disease incidence are 0 and 100%). The statistical fit of the models to the data was evaluated as described before.

Results

Environmental conditions

Moisture conditions, precipitation (≥ 1 mm) and soil moisture tensions at 15 and 30 cm depth, are presented in Fig. 2. The frequency and amounts of precipitation (irrigation not included) between emergence and harvest are given in Table 1. In 1988, soil moisture tensions did not attain extreme values and in irrigated plots tensions were reduced to below 20–30 cbar at 15 cm depth at times when in non-irrigated ones these values were amply exceeded (Fig. 2A). In the summer of 1989, temperatures were relatively high and since irrigation was not always adequately applied in this year, moisture tensions were high also in the irrigated plots. Therefore the difference between irrigated and non-irrigated conditions was not very pronounced (at 15 cm depth) in 1989, although at 30 cm depth the effect of irrigation could be noticed (Fig. 2B). In 1990, irrigation was applied more adequately and differences between irrigated and non-irrigated soil moisture conditions were present almost throughout the season (Fig. 2C). Soil temperatures above 15 °C, at 15 cm depth, were recorded in the beginning of May in 1988 (directly after emergence) and 1990 (two weeks after emergence), and around 13 May (before emergence) in 1989.

Field observations

In 1988, 50% emergence was approximately 11 days after sowing and was regular. The crop developed well. From the end of August, leaves of the plants in plots with the highest inoculum level had a slightly lighter green colour than in the other plots. Root symptoms, such as vascular browning and bearding, were not observed.

Because of weather conditions, sowing was late in 1989, and emergence was delayed and irregular because of the dry top layer of the soil and the absence of precipitation in the first weeks after sowing. Vascular browning in roots was observed already at the first sampling date in June. Yellowing of the leaves started to show around mid July, and more so at higher inoculum levels. In August, most plots had an overall yellow appearance, only those with inoculum levels 0 and 1 had just a few yellow plants. One plant with systemic leaf symptoms was observed in August. Root symptoms were severe in August and September; all types of symptoms were observed, from brown vessels with a yellow to brown discoloration of the surrounding tissue to strong bearding and sometimes rot of the root tip. The early harvest in 1989, resulting in a short growing season, was unintentional but due to the allocation of labour for the manual harvest.

In June 1990, in the non-irrigated strips leaf yellowing was observed, increasing in intensity from the originally non-infested plots towards the plots with the highest inoculum level. Plants in the irrigated strips showed

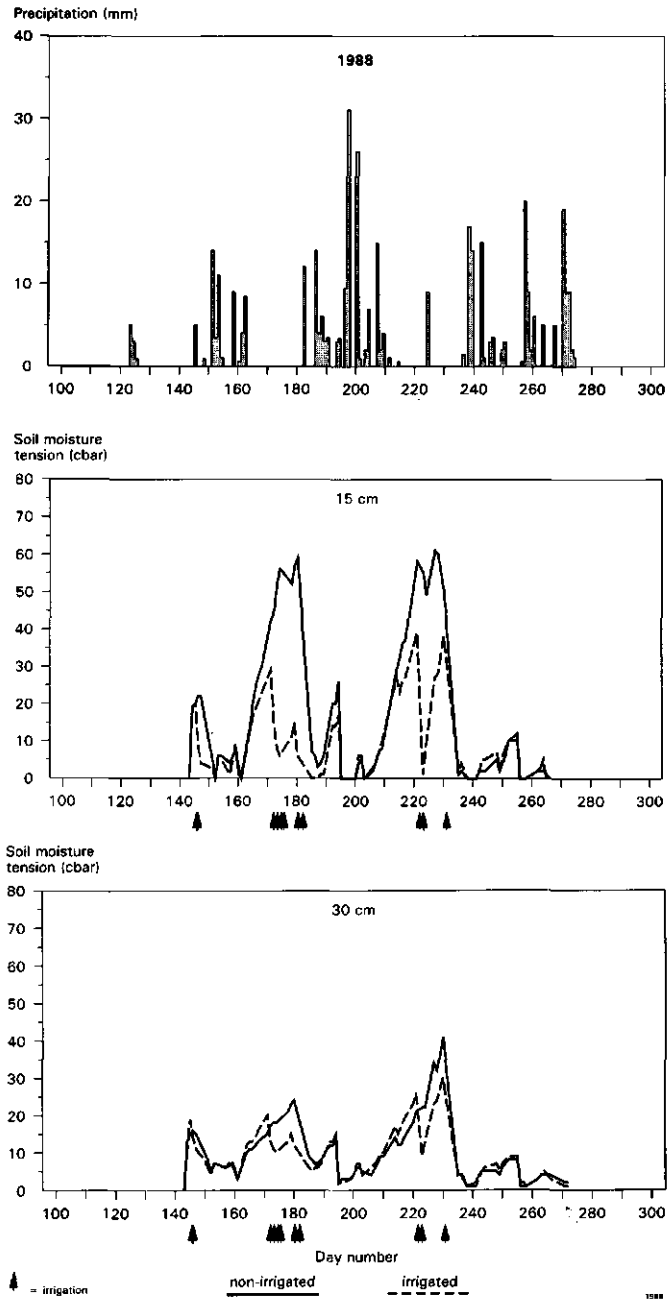

Fig. 2. Amount of precipitation (≥1 mm) per one, occasionally two, days and soil moisture tension (cbar) at 15 and 30 cm depth for the non-irrigated and irrigated plots. Applications of drip irrigation (c. 10 mm each time) are indicated by arrows. A) 1988.

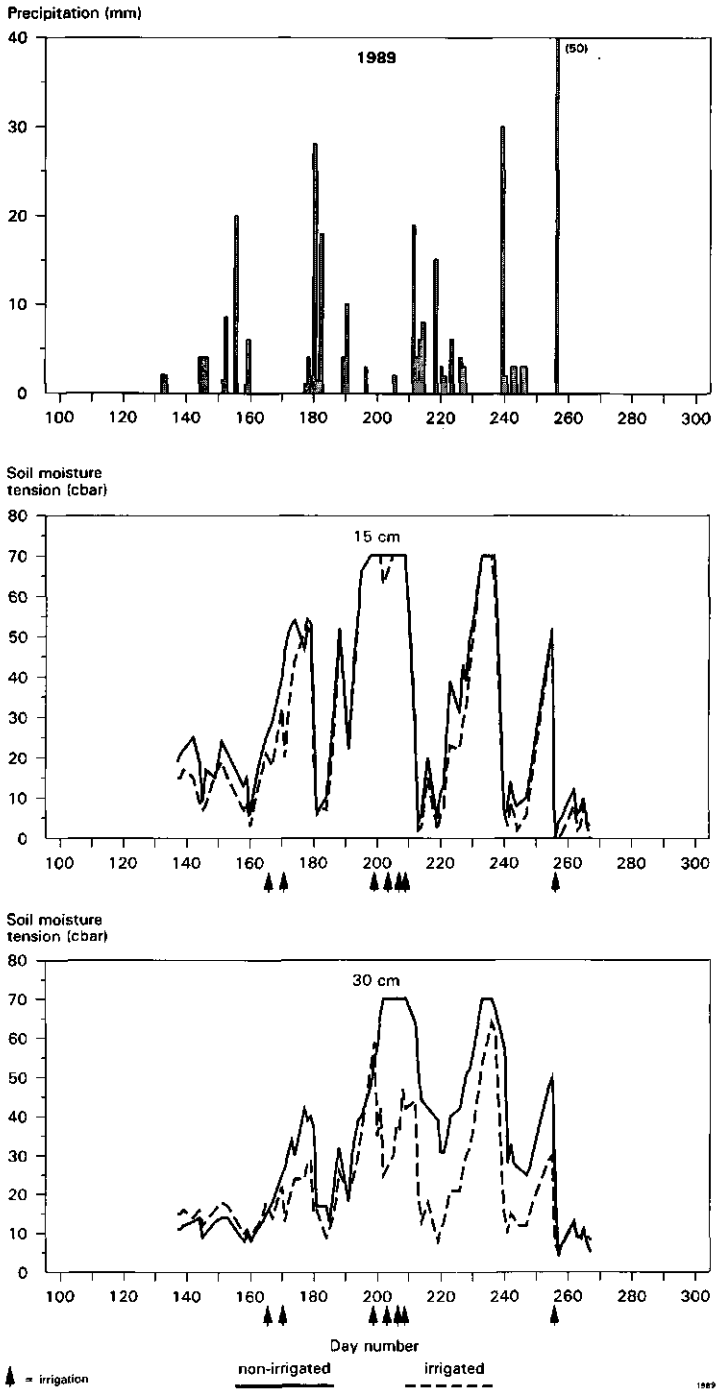

Fig. 2. (Continued) (B) 1989.

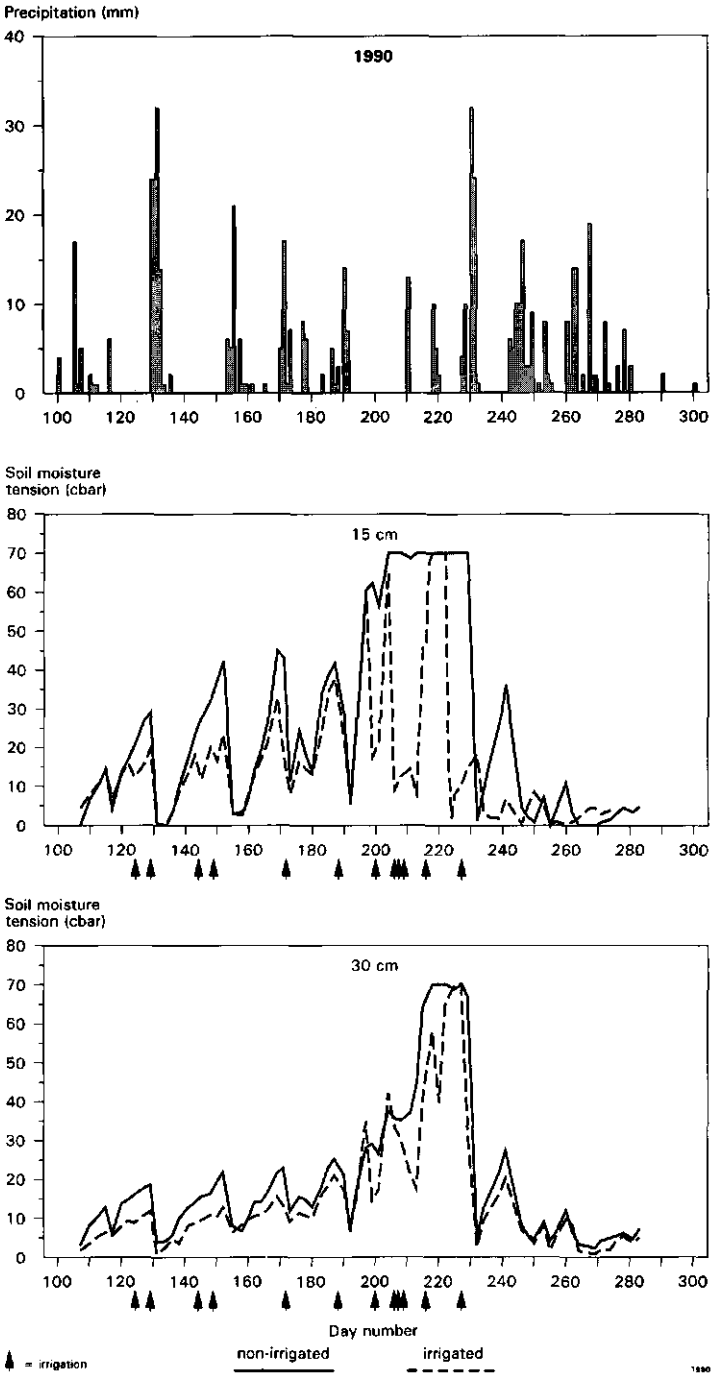

Fig. 2. (Continued) (C) 1990.

a general discoloration of leaves. Other a-typical leaf symptoms, such as a tapering of leaves and upright growth, were observed, as well as several plants with systemic leaf symptoms. Vascular browning in roots occurred in almost all plants of the two highest inoculum levels and in a lower number in the other plots. Bearding of roots was observed on some of these plants in June already. In August and September, high numbers of plants showed both vascular browning and heavy bearding. Plots were 'completely diseased'. From August onwards, leaf infection with *Phoma betae* and *Ramularia beticola* occurred with increasing severity.

Plant development between blocks differed because of differences in soil structure. At the beginning of July 1988, in one complete block (A) soil compaction became visible through water logging after heavy rainfall. In a second block, the same occurred to a minor extent and only in the irrigated strip. In 1989, plants in block A showed more leaf yellowing than plants in the other strips. After a large shower in the beginning of 1990, the whole block was flooded again and plants hardly formed tap roots because of the compact soil. In this year, plants in the irrigated plots of block B were also hampered in their development. Block A was omitted from the analyses of data in all three years.

Contamination of some originally non-infested plots was observed from the end of the first season onwards, the number of contaminated plots and their level of infestation increased during the years [Tuitert and Hofmeester, 1992].

Inoculum of BNYVV in soil increased after one and two beet crops; differences between the originally applied levels diminished. Inoculum levels in 1989 and 1990 differed between non-irrigated and irrigated plots [Tuitert and Hofmeester, 1992]; the effect of irrigation on parameters measured in these years should be considered in combination with the effect of irrigation on inoculum levels in the preceding year(s).

Disease incidence

1988. The first BNYVV-infected plants were detected in July in plots with the highest inoculum level (Fig. 3A). In August, infection was also detected in the irrigated plots of inoculum levels 2 and 3. At harvest, infected plants were found at all levels. The effect of inoculum level on disease incidence was significant ($P < 0.05$). Disease incidence increased in time ($P < 0.001$), with the increase depending on the inoculum level (interaction $P < 0.05$) and on the application of drip irrigation (interaction $P < 0.05$). Disease incidence did not exceed 20%. None of the infected plants showed root symptoms. The few infected plants detected in the control at harvest time (3% in non-irrigated plots, 10% in irrigated plots) were found in the three plots in which BNYVV was also detected in soil samples taken after harvest in October 1988 [Tuitert and Hofmeester, 1992].

1989. Disease incidence was considerably higher in the second year (Fig. 3A, B) than in the first. Already around mid June (six weeks after sowing) high numbers of infected plants were detected (Fig. 3A), up to 90–100% in plots with the highest inoculum level. Disease incidence was higher at higher inoculum levels ($P < 0.001$), was enhanced in the two-year irrigated plots ($P < 0.01$) (Fig. 3B) and increased during the season ($P < 0.01$). The incidence of root symptoms in September showed significant linear correlation (R^2 adjusted 76% with angular transformed data) with the incidence determined by ELISA. In ANOVA of symptom-showing infected plants, a linear effect of initial inoculum level was apparent ($P = 0.055$). The percentage of BNYVV-infected plants (back-transformed angular means) that exhibited root symptoms increased from 50–80% at the lowest inoculum levels to 100% at inoculum level 4. Infected plants were detected in the three control plots already found to be contaminated in 1988, and in one additional plot.

1990. In the third year, the whole field was heavily diseased; high incidences, accompagnied by root symptoms, were recorded by mid June (Fig. 3A). Disease incidence was still related to the initial inoculum level applied ($P < 0.001$), but, as was confirmed by the significant interaction with irrigation ($P < 0.001$), incidences between inoculum levels were only different for the non-irrigated plots (Fig. 3B). There was still an increase in incidence during the season ($P < 0.001$). In all control plots, infected plants were detected. At the three sampling times, there was a significant linear correlation between root symptom incidence and virus infection detected by ELISA; R^2 adjusted was 61, 51 and 71% for the first, second and third sampling, respectively. Addition of the factor initial inoculum level in stepwize regression resulted in a significant increase in the percentage of variance accounted for. The explanation for this and for the deviation from linearity was the finding that, especially at the first sampling, the percentage of BNYVV-infected plants showing root symptoms was higher at higher inoculum levels. In ANOVA of angular transformed percentages of virus-infected plants showing root symptoms, there was a significant linear effect of inoculum level ($P < 0.001$); back-transformed mean percentages increased from 16% for inoculum levels 0 and 1 to 100% for inoculum level 4 at the first sampling date. At harvest these figures ranged from 53% to 100% for the non-irrigated plots of inoculum level 0 and 4, with a mean of 98% for all the irrigated plots.

Root weight, sugar content and sugar yield

1988. In the first year, sugar content was reduced as a result of the applied infestation, but root weight was not significantly influenced (Table 2, Fig. 4A, B). The reduction in sugar content was significant from the first sampling time onwards (Fig. 5B). The mean sugar content at inoculum

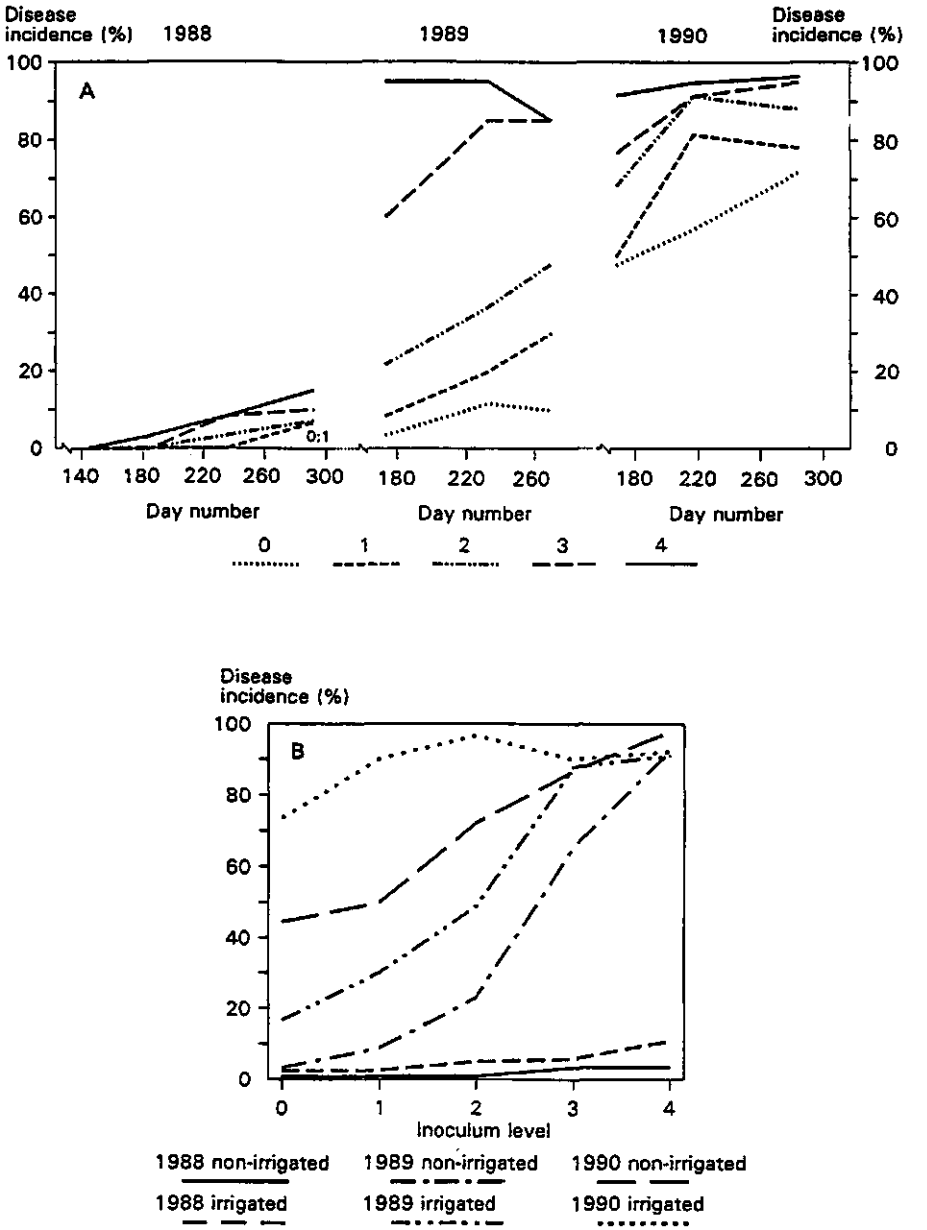

Fig. 3. A) Disease progress curves for the five initial inoculum levels (0–4) in 1988, 1989 and 1990. Arithmetic mean disease incidence (percentage of BNYVV-infected plants detected by ELISA) is plotted. B) Disease incidence, the arithmetic mean of all sampling times, for non-irrigated and irrigated treatments separately.

Table 2. Results of the analysis of variance of yield and quality parameters of sugar beet at different sampling times during three successive years. The trial had a split-plot design, with irrigation levels (two) as main plots and inoculum levels of rhizomania (five) as subplots

Parameters[a]	Significance[b] of factors [c] in ANOVA									Orthogonal polynomial contrasts[d]					
	Early sampling			Intermediate sampling			Harvest time			Harvest time					
	I	R	IxR	I	R	IxR	I	R	IxR	R-lin	R-quad	R-dev	IxR-lin	IxR-quad	IxR-dev
1988	4 July (80)[e]			23 August (130)			18 October (186)								
root weight	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
sugar content	ns	***	ns	ns	***	***	ns	***	*	***	***	ns	***	ns	ns
sugar yield	ns	ns	ns	ns	*	ns	ns	**	ns	**	**	ns	ns	ns	ns
Na	ns	ns	*	ns	ns	ns	ns	**	ns	***	ns	ns	ns	ns	ns
α-amino N	ns	ns	*	ns	ns	ns	ns	***	ns	***	***	ns	ns	ns	*
1989	22 June (68)			21 August (111)			27 September (148)								
root weight		n.d.		***	*	ns	ns	***	*	***	ns	ns	ns	**	ns
sugar content				ns	***	ns	ns	***	ns	***	ns	ns	*	ns	ns
sugar yield				***	**	ns	**	***	*	***	ns	*	*	*	ns
Na				ns	***	ns	ns	***	ns	***	ns	ns	ns	ns	ns
α-amino N				*	*	ns	*	***	***	***	ns	ns	***	ns	ns
1990	18 June (77)			6 August (126)			12 October (193)								
root weight	*	***	*	ns	ns	*	ns	***	***	***	ns	ns	***	ns	ns
sugar content	*	*	ns	ns	*	ns	ns	*	ns	***	ns	ns	**	ns	ns
sugar yield	**	***	*	*	*	*	*	***	***	***	ns	ns	***	ns	ns
Na	**	ns	ns	ns	**	ns	ns	*	ns	**	ns	ns	ns	ns	ns
α-amino N	ns	ns	ns	ns	ns	ns	ns	ns	**	*	ns	ns	***	ns	ns

[a] Units of measurements: fresh weight of root and sugar yield in kg ha^{-1}, sugar content as a percentage of fresh weight of root; contents of Na and α–amino N in mmol kg^{-1} fresh weight of root. At harvest all plants from an area of 20 m^2 were taken for the assessments. At the periodical sampling dates 20 plants were sampled, root weights were converted to weights per ha by means of the plant density of each plot. [b] Significances: *** = $P \leq 0.001$; ** = $P \leq 0.01$; * = $P \leq 0.05$; ns = not significant. [c] Factors: I = Irrigation; R = Rhizomania inoculum level; IxR = interaction between irrigation and inoculum level for one (1988), two (1989) or three (1990) years. [d] Orthogonal polynomial contrasts were used to examine trends in data: linear (lin) and quadratic (quad) effects of inoculum level (R) were examined, variance not accounted for by these effects was assigned to deviations (dev). [e] ( ) number of days after sowing. n.d.: not determined.

level 4 was reduced by 5% in June and 11% in October, compared to the control. Irrigation significantly influenced the linear effect of inoculum level; in the control and the lowest inoculum level sugar content was not affected or slightly increased, from level 2 onwards it was decreased by irrigation (Fig. 4B). The resulting sugar yield was significantly influenced by inoculum level, the reduction by and interaction with irrigation was not significant. Only at the highest inoculum level sugar yield was reduced (by 10%) compared to the control (Fig. 4C and 6), in October.

1989. Root weight decreased progressively with increasing inoculum level in 1989, both in August and September (Tables 2 and 3, Fig. 5A). The effect of two years of irrigation was detected in August as an overall reduction, in September the effect of irrigation depended on the inoculum level. Root weights ranged from 70 and 63 ton ha^{-1} for the non-irrigated and irrigated control, to 32 and 26 ton ha^{-1} for both highest inoculum levels. Sugar content showed a progressive decrease with increasing inoculum level, which was found both in August and September (Fig. 4B). Between the two sampling times, mean sugar content of the highest inoculum level increased with only 0.4%, that of the control with 1.5% (Fig. 5B). Two years of irrigation affected the linear relationship of sugar content with inoculum level (Table 2). The effect of rhizomania on sugar yield was disastrous in this year, where the highest inoculum levels resulted in only about 3000 kg ha^{-1}, a mean reduction of 66% compared to the control plots (Fig. 6). The strong decrease in sugar yield with increasing inoculum level (linear + nonlinear) was enhanced, to a degree depending on the inoculum level, by two-year irrigated conditions (Table 2, Fig. 4C).

1990. The build-up of inoculum at all plots was such that yields obtained in this year were extremely low. Early in the year, root weight still showed a decrease with increasing inoculum level, both for the non-irrigated and irrigated plots (Table 2). At harvest, an effect of inoculum level was detected only for the non-irrigated plots (significant interaction) (Table 2, Fig. 4B), the irrigated plots attained a mean yield level of only 33 ton ha^{-1} (Table 3). In the two periodical samplings, sugar content decreased as inoculum level increased, with an extra decrease in the three-year irrigated plots in June. Sugar content hardly increased between August and October (Fig. 5B). At harvest, there was still a significantly linear effect of inoculum level (Table 2). Effects of inoculum levels and three years of irrigation on root yield were reflected in the effects on sugar yield (Table 2). The increase in sugar yield during the season is presented in Fig. 5C. At harvest, sugar yields ranged from 6587 to 4133 kg ha^{-1} for the non-irrigated plots (Fig. 4C), with an overall mean of 3323 kg ha^{-1} for the irrigated plots (Table 3).

Table 3. Final root weight, sugar content and sugar yield of susceptible sugar beet at different levels of inoculum of BNYVV, without or with irrigation in three successive years. Means of factors are presented for all three parameters, irrespective of the significance of main effects and interactions, as given in Table 2

Year	Inoculum level[a]					Irrigation[b]		Overall mean
	0	1	2	3	4	–	+	
Root weight (× 1000 kg ha^{-1})								
1988	66.17	71.32	67.35	68.90	66.91	73.03	68.13	68.13
1989	66.62	61.32	46.88	35.97	29.41	53.34	42.73	48.04
1990	43.28	43.47	40.49	38.69	35.94	47.73	33.02	40.37
Sugar content (%)								
1988	16.63	16.56	16.29	15.77	14.71	16.18	15.80	16.00
1989	13.38	12.92	11.90	10.79	10.41	12.20	11.56	11.88
1990	11.28	10.83	10.48	10.27	10.28	11.16	10.10	10.63
Sugar yield (kg ha^{-1})								
1988	11002	11809	11005	10899	9871	11813	10022	10917
1989	8932	7952	5624	3873	3051	6722	5050	5886
1990	4959	4775	4305	3964	3696	5356	3323	4340

[a] Initial inoculum levels: 0 = non-infested; 1 to 4 = levels increasing with tenfold steps.
[b] Drip irrigation applied (+) or not (–) during one, two or three successive years, for 1988, 1989 and 1990, respectively.

Quality parameters

1988. Already in the first year, Na content of the roots at harvest increased with increasing inoculum level (Tables 2 and 4), although values were low. At the first sampling in July, the increase was already noticeable in the irrigated plots, but not in the non-irrigated ones, as confirmed by the significant interaction of inoculum level and irrigation. In August, differences were not significant. Mean Na-content at the three sampling dates decreased from 3.95 to 2.11 to 1.66 mmol kg^{-1} root. Alpha-amino N content showed a significant decrease with increasing inoculum level at harvest (Tables 2 and 4). Contents in the irrigated plots were lower than in the non-irrigated ones, but the interaction was not significant ($P = 0.09$). In July an interaction was seen, which did not reflect any clear trend, in August no differences were found. Mean values at the three sampling times were 11.80, 7.77 and 10.98 mmol kg^{-1} root.

1989. Both in August and September, Na content showed a large and significant increase with increasing inoculum level (Table 4, Fig. 7). Values for inoculum level 0 to 4 ranged from 3.80 to 7.58 mmol kg^{-1} root in August and 2.98 to 6.80 mmol kg^{-1} root in September, thus showing up to a 128% increase at the highest inoculum level. Alpha-amino N content

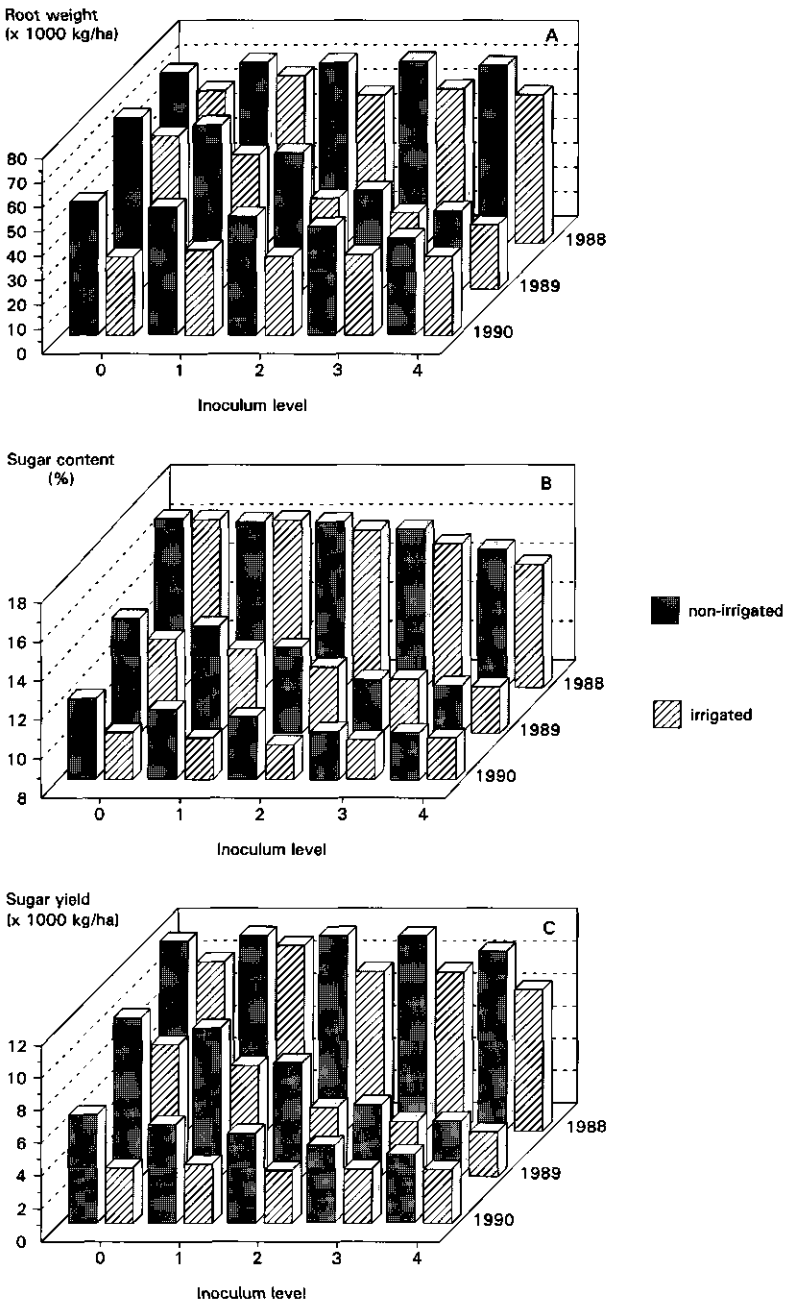

Fig. 4. Yield parameters of sugar beet cv. Regina at five inoculum levels of BNYVV in three successive years, with or without application of drip irrigation. Approximate amounts of BNYVV-infested soil used to create the different inoculum levels in 1988 were 0, 0.5, 5, 50 and 500 kg ha^{-1} for inoculum levels 0–4. See Table 2 for significances of factors and interactions. A. Root weight; B) Sugar content; C) Sugar yield.

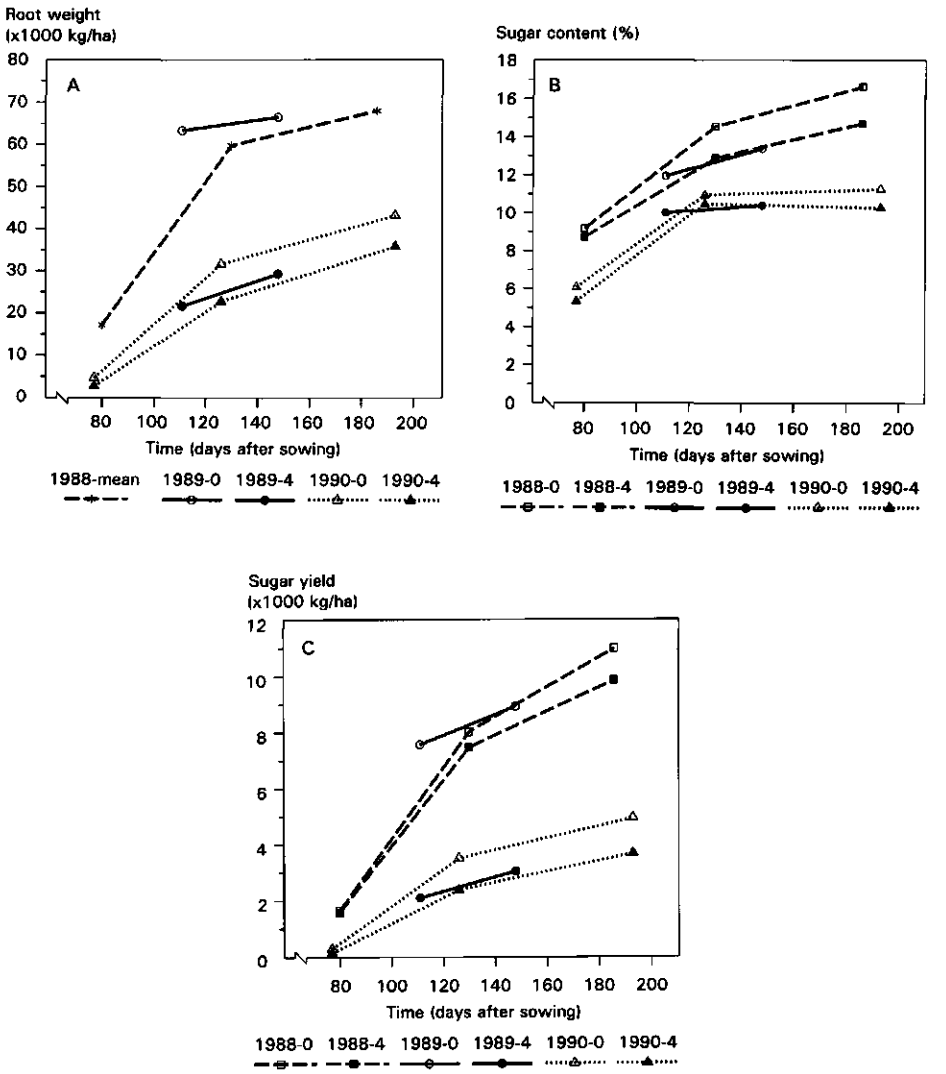

Fig. 5. The increase in yield parameters during the season for the originally non-infested plots (inoculum level 0) and plots with the highest infestation of BNYVV (inoculum level 4) in three successive years. Data are means of non-irrigated and irrigated plots, plotted against number of days after sowing. A) Root weight; B) Sugar content; C) Sugar yield.

reacted distinctly to the different lower inoculum levels, discriminating the two-year irrigated plots from the non-irrigated ones, but at higher inoculum levels the contents leveled off (Fig. 8). In August these effects were also observed, but less pronounced. In August, values ranged from 9.97 to 5.83 mmol kg^{-1}, in September from 7.80 to 3.27 mmol kg^{-1} for inoculum levels 0 to 4, non-irrigated.

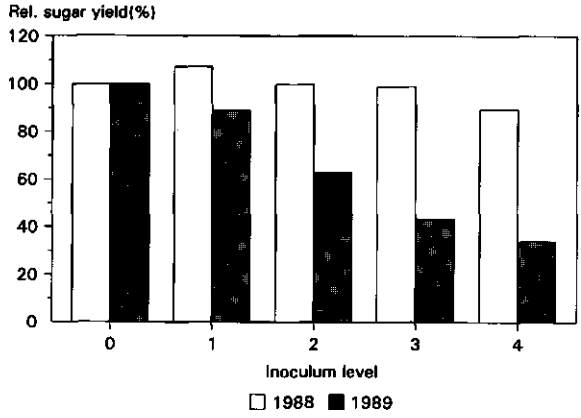

Fig. 6. Mean sugar yield of beet cv. Regina at different inoculum levels of BNYVV in soil, relative to the sugar yield obtained on non-infested control plots, in 1988 and 1989.

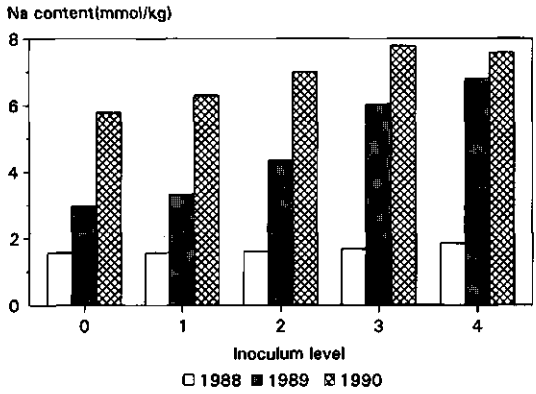

Fig. 7. Mean sodium content in fresh beet root, cv. Regina, at five inoculum levels of BNYVV, in three successive years. The effect of initial inoculum level on Na content was significant in all three years (Table 2).

1990. Early in 1990, Na content was increased by irrigation. Later, in August and October, Na content increased with increasing inoculum level. Mean values at the three sampling times were 15.18, 5.65 and 6.89 mmol kg^{-1}. For α-amino N content, the interaction between inoculum level and irrigation was significant at harvest; a linear trend was present and more so under non-irrigated conditions. Mean values during the year were 15.82, 7.23 and 5.45 mmol kg^{-1}.

In Fig. 9 the relative contents of Na and α-amino N of the four infested treatments as compared to the originally non-infested control at harvest are shown. Na content showes a stronger reaction to increasing inoculum level than α-amino N.

Table 4. Contents of Na and α–amino N in the roots of susceptible sugar beet at harvest, at different levels of inoculum of BNYVV, without or with irrigation in three successive years. Means of factors are presented for both parameters, irrespective of the significance of main effects and interactions, as given in Table 2

Year	Inoculum level[a]					Irrigation[b]		Overall mean
	0	1	2	3	4	–	+	
Na (mmol kg^{-1} root)								
1988	1.57	1.57	1.62	1.68	1.85	1.57	1.74	1.66
1989	2.98	3.33	4.35	6.03	6.80	4.16	5.24	4.70
1990	5.79	6.30	7.00	7.78	7.58	5.78	8.00	6.89
α-*amino N* (mmol kg^{-1} root)								
1988	11.92	11.93	11.70	10.72	8.65	12.17	9.79	10.98
1989	6.07	5.32	4.03	3.53	3.38	5.27	3.66	4.47
1990	6.12	5.72	5.33	5.08	5.00	6.30	4.61	5.45

[a] Initial inoculum levels: 0 = non-infested; 1 to 4 = levels increasing with tenfold steps.
[b] Drip irrigation applied (+) or not (–) during one, two or three successive years, for 1988, 1989 and 1990, respectively.

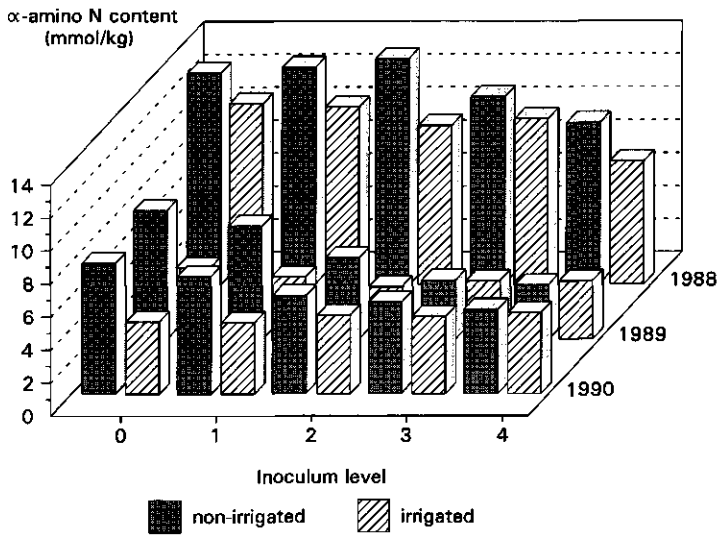

Fig. 8. α-Amino nitrogen content in fresh beet root, cv Regina, at five inoculum levels of BNYVV, in three successive years, with or without application of drip irrigation. See Table 2 for significance of factors and interactions.

Correlation of sugar content and Na content of the root

The negative correlation between sugar and Na content of the root has been recognized as a feature of rhizomania disease [Heijbroek, 1989]. Therefore

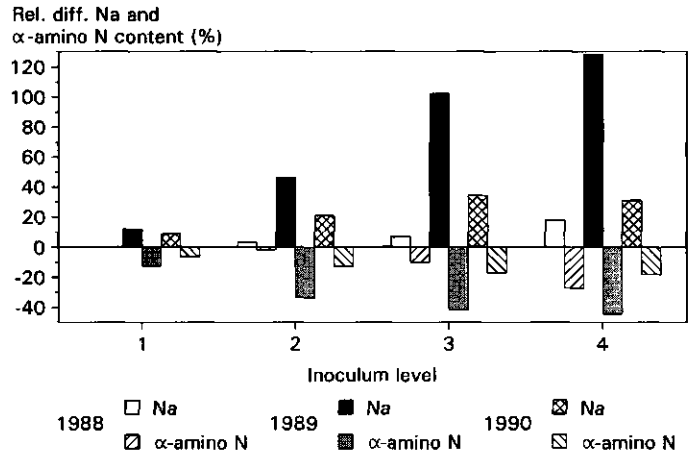

Fig. 9. Relative differences in sodium and α-amino nitrogen content in fresh beet root in plants at different inoculum levels of BNYVV, compared to the contents in plants of the originally non-infested control plots in each year.

the relationship between these parameters was investigated with regression analysis, comparing a linear, a polynomial (linear + quadratic) and an exponential model. Although in 1988 disease severity was low, a linear relationship between the parameters was present (R^2 adjusted 64%, $P < 0.001$). A wide range of inoculum and disease levels was present in 1989, the two quality parameters were highly correlated in this year (R^2 adjusted 94%, $P < 0.001$, for an exponential relationship). In 1990, severity was high for all plots; for the small range of sugar contents in this year the relationship was less close, and best described by an exponential curve (R^2 adjusted 69%, $P < 0.001$). Coefficients of determination from the linear correlation matrix of parameters were –0.81, –0.95 and –0.81 for 1988, 1989 and 1990, respectively. Data of the three years showed a continuous decrease (Fig. 10). For α-amino N the coefficients of determination for linear correlation with sugar content were 0.64, 0.72 and 0.37 for 1988, 1989 and 1990, respectively.

Relationship between MPNs of BNYVV in soil in spring and yield parameters of sugar beet at harvest

1989. The relationship between $\log_{10}$ MPNs of BNYVV and root weight, sugar content and sugar yield was best decribed by Gompertz curves ($P < 0.001$). The effect of irrigation was determined by strips (main plots) and not by individual plots, because of the split-plot design. In ANOVA of regression parameters per strip, for sugar yield only the difference in upper asymptote (A) was significant ($P < 0.05$), whereas for root weight and sugar content differences between parameters for the non-irrigated and

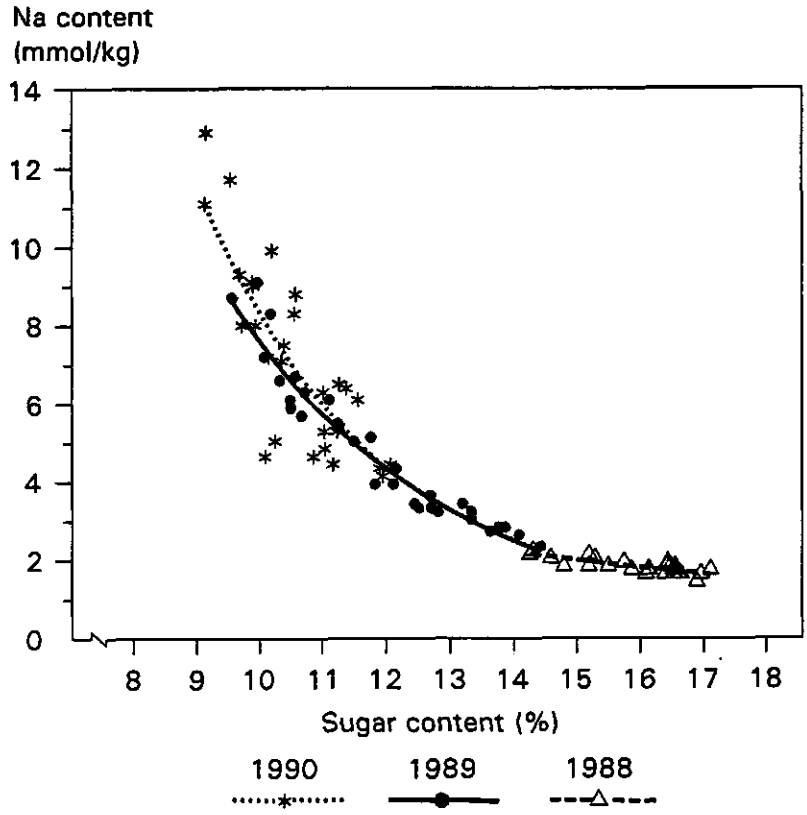

Fig. 10. The relationship between sodium content (mmol kg^{-1} fresh root) and sugar content (% of fresh root) in sugar beet cv. Regina grown on BNYVV-infested plots in three successive years. Regression analysis was performed per year. Regression equations were:
1988: $Y = 4.62 - 0.185\ X$, R^2 adjusted = 64%, $P < 0.001$;
1989: $Y = 1.24 + 401 * 0.66^X$, R^2 adjusted = 94%, $P < 0.001$;
1990: $Y = 3.58 + 7377 * 0.48^X$, R^2 adjusted = 69%, $P < 0.001$.

irrigated set were not significant. For root weight and sugar content the best curve for the pooled data is presented (Fig. 11A, B). For sugar content the logistic model gave the same fit as the Gompertz model. In Fig. 11C the best fitted lines for sugar yield (non-irrigated and irrigated separately) are presented. The considerations with respect to the estimation of the asymptotes and the use of MPN = 0 for inoculum levels below the detection level in bioassay should be kept in mind.

1990. For the three-year irrigated plots, there was no significant relationship between the inoculum potential in soil and yield parameters. For the non-irrigated plots the logistic and Gompertz equations were not appropriate. At the high inoculum levels occurring in this year (log_{10} MPNs

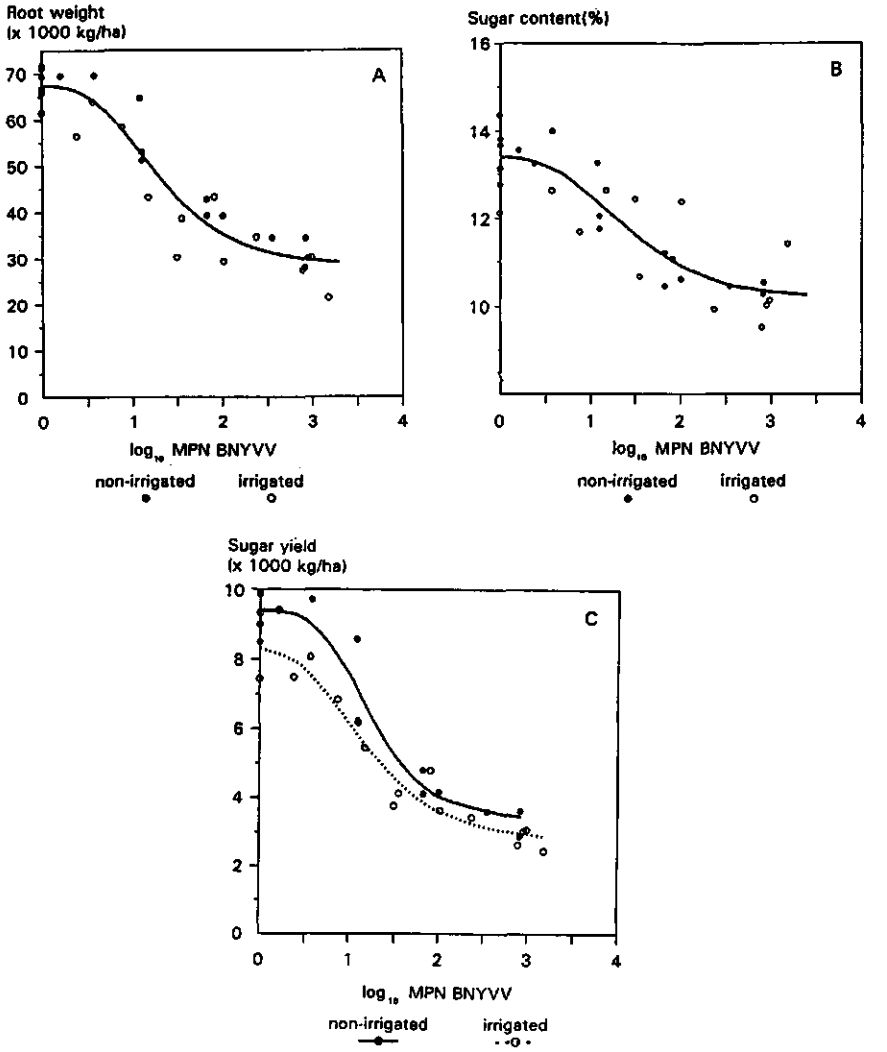

Fig. 11. Yield parameters of sugar beet cv. Regina in relation to the inoculum potential of BNYVV in soil in 1989. Data of all individual plots were used for regression analysis. The inoculum potential is the log-transformed most probable number of infective units per 100 g soil. The Gompertz equation, $y = A + C*\exp(-\exp(-r_g*(x - M)))$, fitted the data best. The effect of irrigation was tested by ANOVA of the equation parameters for each strip separately. For root weight and sugar content the effect of irrigation was not significant (at $P = 0.05$) and one line for the pooled data is drawn, for sugar yield the difference in upper asymptote A was significant ($P < 0.05$), best lines for the non-irrigated and irrigated data set are drawn.

A) Root weight: $A = 67.5$, $r_g = 1.77$, $C = -39.0$, $M = 1.08$, R^2 adjusted = 88%, $P < 0.001$.

B) Sugar content: $A = 13.36$, $r_g = 1.55$, $C = -3.22$, $M = 1.19$, R^2 adjusted = 73%, $P < 0.001$.

C) Sugar yield: non-irrigated $A = 9443$, $r_g = 2.38$, $C = -6078$, $M = 1.08$, R^2 adjusted = 92%, $P < 0.001$; irrigated $A = 8376$, $r_g = 1.77$, $C = -5599$, $M = 0.96$, R^2 adjusted = 92%, $P < 0.001$.

between 1 and 4), root weight and sugar yield showed a linear decrease with increasing soil inoculum (R^2 adjusted 81 and 84%, respectively). The equations for root weight (tons ha^{-1}) and sugar yield (kg ha^{-1}) were: Y = 61.88 – 6.60 * log (MPN BNYVV) and Y = 7582 – 1045 * log (MPN BNYVV), respectively. A slightly higher R^2 adjusted was obtained with the exponential equation (Y = A – B * exp (R * X)), but then standard errors of parameter estimates were very high, amounting up to 1.5 times the estimate itself for B. This was also found for sugar content, were the linear equation, Y = 12.64 – 0.70 * log (MPN BNYVV), explained 62% of the variance.

Relationship between MPNs of BNYVV in soil and disease incidence

1989. Disease incidence recorded as the proportion of infected plants determined by ELISA showed a logistic increase with increasing inoculum potential of BNYVV in the soil for all three sampling times (Table 5, Fig. 12A). The logistic rate of increase and the log MPN value at the point of inflection (Y = 0.5) both were highest in June and decreased in the later samplings. Incidence of plants with root symptoms at harvest could also be described by the logistic equation (Fig. 12B).

1990. Only for the very early infected plants, showing root symptoms already in June, a logistic increase (R^2 adjusted 75%) was found. For all other assessments an increasing trend was present, but the different equations could not explain more than 60% of the variance (Table 5, Fig. 12C, D).

Discussion

Yield of sugar beet at different inoculum levels of BNYVV in soil

Inoculum levels that could hardly be detected in soil by bioassay directly after application of the inoculum to the field [Tuitert and Hofmeester, 1992] caused a reduction of sugar content already in the first year, but had no effect on root weight. In the absence or at the lowest inoculum level of BNYVV, irrigation did not affect sugar content, but at the higher inoculum levels it caused an extra reduction. At the highest inoculum level (0.01% of infested soil calculated to the upper 30 cm of the soil), sugar yield was reduced by 10% compared to the non-infested control. The effect of irrigation, periodically decreasing (positive) soil moisture tensions, will primarily be due to an effect on the vector *P. betae*, enabling more primary infections and more secondary infection cycles, resulting in an enhanced virus content of and spread in the roots. The higher inoculum potentials in the irrigated plots than in the non-irrigated plots after one year [Tuitert and

Table 5. Summary of nonlinear regression parameters and statistics of different models fitted to describe disease incidence (proportion) with increasing inoculum potential ($\log_{10}$ (MPN BNYVV/100 g + 1)) at different sampling times

Model[a]	BNYVV-infected plants, determined by ELISA				Plants showing root symptoms			
	R^2 adj. (%)[b]	Rate ± s.d.[c]		M or B[d]	R^2 adj. (%)[b]	Rate ± s.d.[c]		M or B[d]
1989: June								
Logistic	87	2.78 ± 0.56		1.96	n.d.			
Gompertz	84	1.88 ± 0.39		1.73				
Monomolecular	68	0.64 ± 0.05	(0.45)	1.08				
1989: August								
Logistic	79	2.00 ± 0.39		1.44	n.d.			
Gompertz	77	1.27 ± 0.25		1.09				
Monomolecular	71	0.55 ± 0.05	(0.59)	0.99				
1989: September								
Logistic	81	1.84 ± 0.30		1.29	89	1.98 ± 0.28		1.52
Gompertz	80	1.20 ± 0.19		0.93	89	1.41 ± 0.20		1.21
Monomolecular	76	0.55 ± 0.05	(0.60)	0.95	80	0.57 ± 0.04	(0.56)	1.02
1990: June								
Logistic	60	1.61 ± 0.36		2.00	75	2.81 ± 0.61		2.54
Gompertz	58	1.14 ± 0.25		1.60	74	1.88 ± 0.39		2.30
Monomolecular	54	0.50 ± 0.07	(0.70)	1.71	59	0.54 ± 0.06	(0.61)	2.07
1990: August								
Logistic	42	1.25 ± 0.33		0.97	56	1.31 ± 0.28		1.35
Gompertz	42	1.08 ± 0.28		0.65	57	1.08 ± 0.21		1.00
Monomolecular	42	0.40 ± 0.09	(0.92)	1.27	57	0.42 ± 0.06	(0.87)	1.55

Table 5. (Continued)

Model[a]	BNYVV-infected plants, determined by ELISA				Plants showing root symptoms		
	R^2 adj. (%)[b]	Rate ± s.d.[c]		M or B[d]	R^2 adj. (%)[b]	Rate ± s.d.[c]	M or B[d]
1990: October							
Logistic	50	1.19 ± 0.28		0.72	58	1.44 ± 0.31	1.47
Gompertz	49	1.04 ± 0.24		0.40	56	1.11 ± 0.23	1.07
Monomolecular	48	0.41 ± 0.08	(0.90)	1.02	54	0.43 ± 0.07	1.50

[a] The models were fitted using the FITNONLINEAR directive of GENSTAT 5 [Payne et al., 1988]. The following equations were used: $y = (1 - B*R^x)$ for the monomolecular model, with B the integration constant and the general form of the monomolecular rate r_m equivalent to –log(Rate) and presented in brackets. The logistic equation used was $y = A + C/(1 + \exp(-r_l(x - M)))$, with the upper and lower asymptote set to 0 and 1 by setting A = 0 and C = 1, r_l the logistic rate of increase and M the point of inflection for the explanatory variable ($\log_{10}$ MPN BNYVV). For the Gompertz equation, $y = A + C * \exp(-\exp(-r_g(x - M)))$, A and C were also set to 0 and 1, respectively, r_g was the rate of increase and M the point of inflection. All regressions were significant ($P < 0.001$), there were no differences between non-irrigated and irrigated plots.

[b] The percentage of variance accounted for by the model.

[c] The rate parameters of the different models are presented with their standard deviations. For the monomolecular model rate (r_m), in the equation $y = 1 - B * \exp(-r_m * x)$, is given in brackets.

[d] For logistic and Gompertz equations the points of inflection (M) are given, for the monomolecular equations the integration constant (B).

n.d.: not determined.

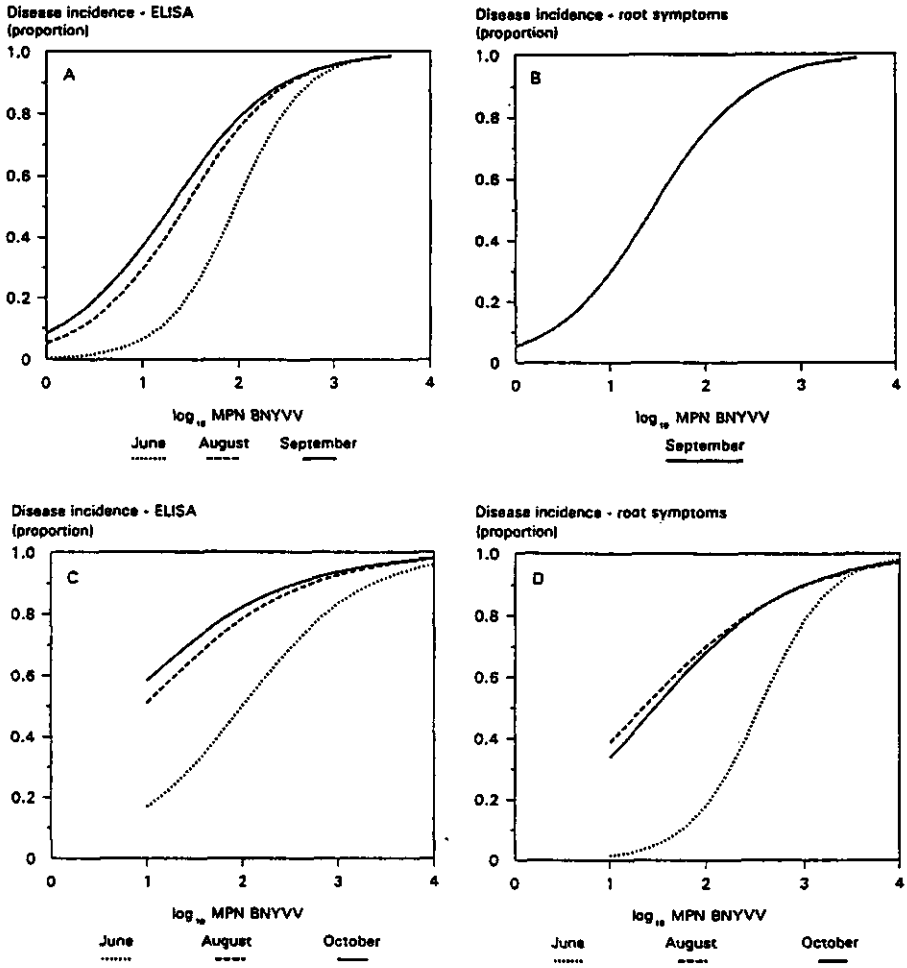

Fig. 12. The logistic relationship between the inoculum potential of BNYVV in soil (log most probable number of infective units per 100 g soil) in spring and disease incidence at different sampling times. Disease incidence (proportion) was determined by ELISA of root tips (A and C), or by visual rating of tap roots for the presence of symptoms (B and D). Parameters of the equations and some statistics are given in Table 5. A) 1989-ELISA; B) 1989-root symptoms; C) 1990-ELISA; D) 1990-root symptoms.

Hofmeester, 1992] support the statement on the irrigation effect. Other effects of irrigation relevant to the infection of roots were discussed earlier [Tuitert and Hofmeester, 1992].

In the first year, multiplication of inoculum in soil [Tuitert and Hofmeester, 1992] was such that in the next year, 1989, both sugar content and root yield decreased progressively with increasing inoculum level. Sugar yield reductions of 11% up to 66% at the highest inoculum level occurred. Two years of extra moist conditions aggravated the reduction of

sugar yield in the second year, especially at the lower inoculum levels. The effect of irrigation should mainly be ascribed to the higher infestation of irrigated plots of the same initial inoculum level, caused by irrigation in 1988 [Tuitert and Hofmeester, 1992]. An additional reduction by irrigation in 1989 apparently occurred at the lower inoculum levels (Fig. 11), remarks on the statistical analysis were made before. Since timing and frequency of irrigation during the summer of this year were not adequate to maintain a higher moisture content in the irrigated plots compared to non-irrigated ones for longer periods of time, effects of irrigation could have been stronger.

After two successive years, BNYVV-infestation had increased to high levels and all control plots had become contaminated. For the non-irrigated plots, root weight and sugar yield still showed a decrease with increasing inoculum level, for the three-year irrigated plots the mean sugar yield was 3300 kg ha^{-1}. At the highest inoculum level this low yield level (around 30% of the disease-free yield in 1988) had already been recorded in the second beet crop after infestation of the plots.

In climate room experiments, BNYVV affected root weight at lower inoculum levels than in the field. In an 18-week bioassay using small pots, tap root weights of plants growing in soil dilution 10^{-4}, comparable to inoculum level 4 in the field in 1988, were reduced by 50% [Tuitert and Bollen, 1993]. In the field, only sugar content was reduced, by 9–14%, at this infestation level. Results of greenhouse and climate room experiments may be more pronounced than those of field experiments for several reasons: optimal temperatures are maintained, water is supplied regularly, inoculum will be uniformly distributed in the soil, the pot volume limits the extension of the root system, and the soil volume will be more densely explored by the roots. In greenhouse experiments with large pots and a three-month growing period, the effects of a lowest soil dilution of 10^{-3} on sugar content and root yield [Bürcky et al., 1986] corresponded with those of our field experiment.

Müller and Gößwein [1987] studied the influence of irrigation on rhizomania in naturally infested fields. Twice they found that irrigation increased sugar content, twice a decrease was observed. Root weight was increased by irrigation in a relatively dry year, but was not affected by irrigation in a year with sufficient precipitation. No information on the degrees of infestation of the experimental fields was given and disease incidence recordings were not presented. Sugar beet is moderately tolerant to low soil moisture content [Van der Schans and Drenth, 1989], the severity of effects of drought depending on the growth stage at which drought is experienced [Brown et al., 1987]. Under drought stress conditions, in the absence of rhizomania, irrigation treatments increase root weight, but have little effect on sugar content [Winter, 1988; Davidoff and Hanks, 1989; Van der Schans and Drenth, 1989]. When irrigation is applied shortly before harvest, hydration effects may cause a lower sugar content on fresh weight basis.

In 1988, with sufficient precipitation, irrigation did not significantly affect root weight; neither in the control, nor in the infested plots. In the relatively dry second year, an increase in root yield in the absence of disease could have been expected. However, the slight contamination of the control plots in 1989 was probably responsible for the negative effect of irrigation on root weight also in these plots.

In the soil used to infest the plots no other pathogens were observed. The correlation of yield and damage with the amount of infested soil added can be ascribed to BNYVV, as was apparent also from the correlation of infestation levels with disease incidence. Effects of the vector itself on yield under field conditions have not been reported. Initial inoculum levels, created by application of tenfold different amounts of infested soil, did not result in tenfold differences in *P. betae* inoculum, because of the presence of a relatively high resident *P. betae* population [Tuitert and Hofmeester, 1992]. Therefore, vector populations could not be responsible for the described effects. The presence of other soil-borne viruses of sugar beet can not be excluded, however, these viruses have not been shown to cause rhizomania symptoms or yield reductions in the field [Henry et al., 1986; Lesemann et al., 1989; Büttner, 1992]. The occurrence of beet soil-borne virus (BSBV) in the Netherlands has not been investigated. Beet cyst nematodes were detected neither in the infested soil added, nor in soil samples taken from the plots before and after the first and second beet crop. In the absence of beet cyst nematodes (or BNYVV), sugar beet has been grown in monoculture for 3 [Heijbroek and Van de Bund, 1982] up to 6 [Lamers, 1981] years without a reduction in yield level. The lower yield in the control plots in 1989 and 1990 compared to 1988 can mainly be ascribed to a short growing season (1989) and to contamination of these plots by BNYVV (1989 and 1990).

Yield parameters measured were all fresh weights. The effects of BNYVV on the conversion efficiency between intercepted solar radiation and dry matter production, and the effects on dry matter distribution will be published separately [Haverkort et al., unpublished].

The effect of infection with BNYVV on quality parameters of beet

Rhizomania affects the concentrations of sodium, potassium and α-amino nitrogen in the root and equations were described, using these parameters and sugar content, for establishing rhizomania signals [Wieninger and Rösner, 1983; Pollach, 1984]. High concentrations of these compounds negatively influence the extractability of sugar [Van Geijn et al., 1983].

In this field experiment, we found that both Na and α-amino N content were sensitive indicators of rhizomania, showing slight but significant changes at the low infestation levels of the first year. At the higher disease levels in 1989, Na showed the highest relative differences compared to the control with distinguishable values over the whole range of inoculum

levels, whereas α-amino N distinguished between the non-irrigated and irrigated plots at lower levels. As in 1990 all control plots were infested, with a geometric mean inoculum potential corresponding to a value between those of inoculum level 2 and 3 in 1989, the relative differences compared to these plots were smaller than they would have been with disease-free controls. Actually, Na content of inoculum level 0 in 1990 corresponded to a value between the contents at inoculum levels 2 and 3 in 1989.

Na content was highly correlated with sugar content, especially in 1989, and although the relationship was nonlinear, the results for Na confirmed the observations of Heijbroek [1989] for a series of infested fields. For the underlying physiological causes of the increased Na content there are only speculations, as described in a review by Bürcky [1987].

Heijbroek [1989] chose the quotient of Na (mmol kg^{-1} root) and sugar (sucrose, % of fresh weight) as an indicator of rhizomania, mentioning that if this exceeded 0.5 (mmol 10 g^{-1} sugar), rhizomania infestation was present. In our trial, the value of 0.5 was exceeded only in inoculum level 3 and 4 in 1989, in non-irrigated inoculum levels 2, 3 and 4 and in all irrigated levels of 1990; whereas this was not the case in the lower, but diseased, levels in 1989 and 1990, and at the low infestations in 1988. A threshold value of 0.5 was too conservative for this field and also for two naturally infested trial fields at other locations [Tuitert, unpublished]. For non-infested trial fields, ratios of 0.24 and 0.27 were found [Tuitert, unpublished], which exceeded the values for inoculum level 4 in 1988. As for a range of inoculum levels α-amino N content was significantly affected, inclusion of the content of this compound in the formula might add to its indicative possibilities. Division of Na content by the product of α-amino N and sugar content followed by multiplication with a factor of 100, could be applied. In this experiment, treatments with yield reductions had values ≥ 2. Whatever formula is used, determination of a threshold value is difficult; with the formulas from Wieninger and Rösner [1983] and Pollach [1984] (both included K content) all inoculum levels of 1988, and two non-infested trial fields referred to before, exceeded the threshold for rhizomania using the formula from the first-mentioned authors, but were in the range of non-infested fields when using the second one.

Data of potassium contents were not presented, because this parameter is less sensitive to rhizomania than the ones treated before. In 1989 and 1990, however, a general increase in K content was found with increasing inoculum level, but the ranking order of inoculum levels was not always reflected in the order of K contents found. In 1988, K content was lowest in plots with the highest inoculum level. This reverse effect might be explained by the relative insensitivity of K to the very low rhizomania disease levels in the first year, whereby K behaves as in the absence of disease. K and Na are partly exchangeable cations for the sugar beet plant [Kirby et al., 1987]; in fertilization experiments a negative correlation

between K and Na content in the root was observed for Na contents up to approximately 4 mmol kg^{-1} root [Van der Beek and Withagen, 1988].

Modelling yield in relation to inoculum potential of BNYVV in soil

Within the range of MPNs of BNYVV occurring in 1989, a nonlinear relationship was demonstrated between soil inoculum and yield parameters of sugar beet. Gompertz (or logistic) equations were appropriate for describing the relationship between inoculum potential (log_{10} transformed) and root weight, sugar yield and sugar content in this year on this particular field. The finding underlines the importance of initial soil inoculum, and thus primary infection, in determining disease severity, caused by a pathogen whose vector has several secondary infection cycles during the season. The upper sugar yield level where little or no yield loss occurred, partly because of compensation [Zadoks and Schein, 1979], continued to an inoculum level of approximately 3–5 infective units of BNYVV per 100 g of dry soil. The sigmoidal transition phase led to the residual yield level of 3 tons ha^{-1} already referred to, beyond which an increase in inoculum did not have an additional effect. The parameters of the curve will not only depend on the MPNs determined, and environmental influences, but also on the pattern of dispersion of inoculum in the field and the age of the plants at primary infection. Inoculum levels below the detection level of the bioassay (at X = 0.2 in Fig. 11) were considered 0 in the analyses. As even some control plots for which a MPN = 0 was determined were probably contaminated, this may have led to an underestimation of the upper asymptote of the curves, especially for the irrigated plots.

In 1990, inoculum potentials had further increased, all control plots were highly infested and MPNs below 10 infective units per 100 g of soil were not available. The range of inoculum levels was too narrow and too much restricted to high MPNs for investigation of the inoculum – yield relationship. For the non-irrigated plots a significant (linear) decrease in yield parameters with increasing inoculum potential was still present. In the irrigated plots, with even higher MPNs, no significant relationship was present, due in part to the distinct data of block B, which were strongly influenced by bad soil structure in this year.

Disease incidence

Disease incidence was measured by analyzing plants for the presence of BNYVV and by observation of root symptoms on these plants. At the low infestations in 1988, disease incidence – the number of BNYVV-infected plants determined by ELISA – was low and gradually increased in time. The measured increase in disease incidence during the season will have been the result of a) an increased number of infected plants because of an

increase in primary infection of the growing root system of plants exploring a larger volume of soil with increasing root density, and b) the time occurring between primary infection of rootlets and occurrence of BNYVV in the tap root in a concentration that can be detected by ELISA. Irrigation caused an earlier increase in infected plants and two- to threefold higher numbers of infected plants at harvest. The latter effect is in agreement with a mean threefold higher multiplication ratio of BNYVV in soil in the irrigated plots in 1988 [Tuitert and Hofmeester, 1992].

In 1989, disease incidence was determined by the inoculum level in soil at the three sampling times. At harvest, the proportion of BNYVV-infected plants that showed root symptoms was also influenced by the initial inoculum level. The higher proportion of symptomatic plants at high inoculum levels may have been the result of a more extensive, early, primary infection at these levels, whereas an earlier time of infection may also have contributed by allowing a longer period of symptom development. The two-year effect of irrigation on disease incidence was probably mainly due to higher soil infestation (MPNs) caused by irrigation in the first year, as in regression analysis, using the MPNs determined for each plot in 1989, irrigation was not a significant factor.

Disease incidence was very high in all plots in 1990; at inoculum levels (expressed in MPNs) similar to those in 1989, disease incidence was higher in 1990. The latter might partly be due to a more regular dispersion of inoculum after two years of beet growing, whereas the few infected plants in 1988 might have caused an aggregated pattern (randomly dispersed infested patches) of inoculum in 1989 at the same measured inoculum potential. The final level of disease may be lower if soil-borne inoculum is highly clustered than when it is evenly distributed [Campbell, 1986]. Especially at the early sampling date, the number of BNYVV-infected plants that showed symptoms increased with increasing initial inoculum level, as was observed in 1989.

Modelling the relationship between inoculum potential and disease incidence of BNYVV

The relationship between inoculum potential (log MPN) and disease incidence in 1989 fitted a logistic model. The fitted lines shifted to the left during the season, whereby the delay parameter, the X-value where the inflection point of the curve occurs, shifted to lower MPNs. Because the Y-max (upper asymptote) cannot increase above 1 and incidences do increase at low MPNs, the rates of increase in disease incidence with increasing log MPN decrease during the season. The increase in disease incidence can again mainly be ascribed to the different times and intensities of primary infection of the growing root system and to either the time necessary for multiplication of virus in the root to detectable levels or the incubation period to symptom development. Periods for virus multiplication

and symptom development are likely to be variable, because both will depend on primary infection and environmental conditions such as temperature and soil moisture (determining the number of secondary infection cycles). All these factors may have contributed to the fit of a logistic equation for describing the increase of disease incidence with increasing log MPN.

It was hypothesized that, although multiplication of inoculum during the season and the increase of root infection of a single plant are polycyclic processes, development of rhizomania incidence in the field during one season is a monocyclic process [Tuitert, 1993]. The finding that disease incidence at different inoculum levels could better be fitted with a logistic than a monomolecular equation, neither contradicts nor proves the correctness of the hypothesis. Deterministic models (descriptions) such as the logistic, Gompertz or monomolecular models can be used to characterize an epidemic and provide a basis for comparison between epidemics, but no conclusions on the nature of the disease cycle should be inferred from the statistical appropriateness of one of these models [Pfender, 1982].

Disease progress in time could not be modelled because of the limited number of samplings. The relationship found between inoculum density and disease incidence indicates that when disease progress curves in time would be constructed, the inoculum potential of the field has to be taken into account. Maximum disease level cannot be set to 1 (100%) at all inoculum potentials, as this might lead to the underestimation of the rate parameter [Neher and Campbell, 1992].

As the logistic model gave the best fit, an alternative for calculation of curve parameters would have been by means of generalized linear modelling with a logistic link function and declaration of a binomial error distribution of the disease incidence data. Rate parameters thus calculated did hardly differ from the ones presented (Table 5).

Risk of soil displacement

Displacement of infested soil is an important way of spreading disease. The risks attached to small amounts of infested soil are illustrated by the results of the described field experiment. One year of sugar beet may be sufficient to increase hardly detectable inoculum levels to harmful levels. To plots of inoculum levels 2 and 3 about 5 and 50 kg infested soil ha^{-1} was added, which resulted in mean yield reductions of 37 and 57%, respectively, in the second beet crop after infestation of the plots. As the average amount of soil allowed on seed potatoes for 1 ha of land is approximately 20 kg, the potential risk of using planting material from infested fields is obvious.

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Chapter 6

Epidemiology of beet necrotic yellow vein virus in sugar beet at different initial inoculum levels in the presence or absence of irrigation:

Dynamics of inoculum

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Epidemiology of beet necrotic yellow vein virus in sugar beet at different initial inoculum levels in the presence or absence of irrigation: Dynamics of inoculum

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Abstract

Using field plots where rhizomania had not previously been detected, different inoculum levels of beet necrotic yellow vein virus (BNYVV) were created by application of infested soil. A susceptible sugar beet cultivar (cv. Regina) was grown for two consecutive years (1988 and 1989), in the presence or absence of drip irrigation. In soil samples taken in spring 1989, the different initial inoculum levels of 1988 could be distinguished using a quantitative bioassay estimating most probable numbers (MPNs) of infective units per 100 g dry soil. The first sugar beet crop resulted in a ten-thousandfold multiplication of inoculum of BNYVV (viruliferous *Polymyxa betae*). Mean MPNs of BNYVV ranged from 0.6 and 7 per 100 g soil for the lowest inoculum level to 630 and 1100 per 100 g for the highest level, in plots without and with irrigation, respectively. In spring 1990, MPNs had again increased. In both years, the initial inoculum level of 1988 had a significant linear effect on log-transformed MPNs of BNYVV determined. Log-transformed MPNs for 1990 and 1989 showed a positive linear correlation, despite a decreasing multiplication ratio at higher inoculum levels. Drip irrigation during one or two years enhanced the increase in MPN of BNYVV, which was reflected by the enhancement of multiplication ratios at all inoculum levels. The total *P. betae* population was also higher after growing two irrigated crops than after growing two non-irrigated ones.

Additional keywords: *Polymyxa betae*, rhizomania, BNYVV, soil-borne virus, MPN, quantitative technique, bioassay, polyetic epidemic.

Introduction

Beet necrotic yellow vein virus (BNYVV), the causal agent of rhizomania in sugar beet, is transmitted by the fungus *Polymyxa betae* Keskin. The vector is an obligate parasite, which, after primary infection, develops zoosporangia releasing zoospores in a rapid multiplication cycle (Keskin, 1964), and thus has a potentially high multiplication rate. The formation of resting spores, and their longevity, assures its survival in soil between successive beet crops. The epidemiology of BNYVV is largely determined by the behaviour of its vector. Ecological aspects, including the influence of soil moisture and temperature on fungal development, have been studied (Abe, 1987; Asher and Blunt, 1987; Blunt et al., 1991; De Heij, 1991). However, quantitative epidemiology of the disease has received little attention. Studies on the dynamics of BNYVV in soil as influenced by a susceptible beet cultivar have not yet been published.

Field levels of BNYVV infestation have been characterized by: (a) the resulting disease incidence or severity or yield of susceptible cultivars as compared to yield of resistant/tolerant cultivars (Winner, 1988); (b) the number of BNYVV-infected bait plants in a bioassay on soil samples (Beemster and De Heij, 1987) and (c) the ELISA absorbance values for BNYVV in bioassay plants (Hillmann, 1984; Büttner and Bürcky, 1990). Quantitative methods used to assess the vector in soil did not include quantification of BNYVV (Goffart et al., 1987, 1989; Ciafardini and Marotta, 1989), and applications of these methods to study the dynamics of non-viruliferous *P. betae* were not reported. A bioassay procedure based on serial dilutions of BNYVV-infested soil permitted assessment of the inoculum potential of both vector and virus (Tuitert, 1990).

In 1988, a field experiment was set up to examine disease development at different initial inoculum levels of BNYVV, in the presence and absence of drip irrigation. Preliminary reports of the work were given (Hofmeester and Tuitert, 1989; Tuitert and Hofmeester, 1990). This paper presents the results of a study on the dynamics of BNYVV: the quantification of inoculum build-up during two successive beet crops.

Material and methods

Field plot design. In 1988, a field trial was laid out on a calcareous clay soil (pH-KCl 7.4; organic matter 4.2%), located at an isolated site in an urban area in the Noordoostpolder, the Netherlands. Sugar beet had never been grown on this field and BNYVV could not be detected in any of the plots by bioassay of soil samples taken in November 1987.

The experiment was arranged in a split-plot design, with irrigation levels as main plots and inoculum levels as subplots and with four blocks (Fig. 1). The five inoculum level subplots were not randomized within the main plots. In order to reduce the effect of infested soil being moved from one inoculum level to another, subplots were laid out in a fixed order of increasing inoculum level. Between and within blocks the irrigated and non-irrigated main plots were separated by grass strips 3 m wide. Drilling and soil cultivation always started in the uninfested control plot and, to clean the equipment, ended in an extra uninfested area behind the plot with the highest inoculum level. Tillage practices were performed when weather and soil were dry. Machines were cleaned before entering each main plot. As became apparent during the first year, one complete block suffered from water logging because of soil compaction. In view of the poor emergence and development of the plants in all three years the data from this block were not used.

Plot size was 6 x 10 m. Sugar beet cv. Regina was sown in three consecutive years in rows 50 cm apart with a sowing distance of 18.5 cm. Irrigation was applied along every row with a drip irrigation system with emitters spaced 30 cm apart. Soil moisture was recorded by tensiometers three to five times a week at 15 and 30 cm depth. During dry periods, when soil moisture tension (Hillel, 1982: soil water potential presented as a positive value) at 15 cm depth exceeded 20 cbar, drip irrigation was used to supply about 10 mm water during 2–3 hours.

Generating different inoculum levels. Infested soil from a field in the Noordoostpolder with a history of rhizomania was used as inoculum to create plots with different infestation levels. This soil was dried, ground and thoroughly mixed with sterilized river sand in

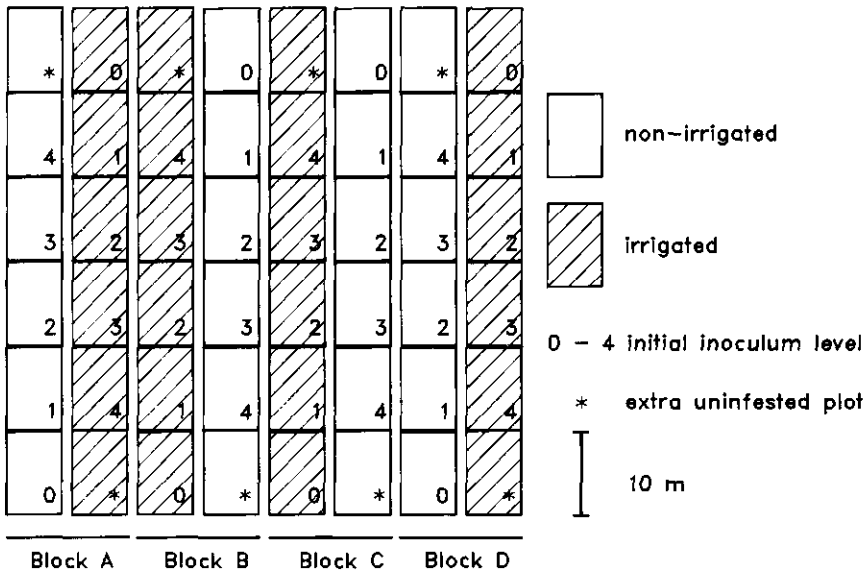

Fig. 1. Layout of the experiment. For explanation of initial inoculum levels see Table 1. Because of water logging, block A has not been used in the analysis.

different proportions. Two days before sowing, the mixtures were applied to the plots (3 kg per plot) by hand and were superficially raked into the soil. Volume ratios of infested soil at the five inoculum levels are given in Table 1.

Soil sampling. Soil samples were composed of 1.3 cm diameter cores taken to a depth of 25 cm in a 1 x 1.4 m grid in every plot (42 cores per sample). Soil was air-dried and ground before use. Mean bulk density of the soil was 1.3 g · ml^{-1}. Samples were collected before (1987) and shortly after introduction of the infested soil to the plots (April 1988) and immediately after harvest (October 1988). In 1989 and 1990 samples were taken shortly after drilling (May).

Assessment of BNYVV and P. betae in soil. Samples collected in 1987 and 1988 were analysed by bioassay for the presence of BNYVV and *P. betae*. In 1987, five pots of 200

Table 1. Amount of BNYVV-infested soil applied per inoculum level in April 1987. The volume ratio was based on a tillage layer of 30 cm.

Initial inoculum level	Amount of BNYVV-infested soil applied	
	Volume ratio (%)	Average amount (g · m^2)
0	0	0
1	0.00001	0.05
2	0.0001	0.48
3	0.001	4.80
4	0.01	48.00

ml were filled with undiluted soil per sample. The dried soil samples of 1988 were mixed with 50% (v/v) of sterile coarse sand. Ten pots of 200 ml were filled with this mixture per sample. In each pot a sugar beet seedling (cv. Regina, 2–3 weeks old) was planted. For this bioassay the detection limit was estimated as 0.08 infective units per 100 g of soil. Samples collected in 1989 and 1990 were serially diluted. Assessments of the inoculum potential of BNYVV in 1989 and of *P. betae* and BNYVV in 1990 were made by the most probable number method (Tuitert, 1990). The soil used to infest the field in 1988 was analysed in the same way, both for *P. betae* and the virus. A dilution ratio of 10 with six replicates per dilution was used. Greenhouse conditions and plant analysis for BNYVV and *P. betae* for both types of assays were as described by Tuitert (1990).

The inoculum potential of BNYVV and *P. betae* in soil was estimated as the most probable number (MPN) of infective units per 100 g of soil. The detection limit of the dilution method was a MPN of 0.6 units per 100 g of soil. Multiplication ratios of BNYVV in 1988 were estimated by dividing the measured MPNs in spring of 1989 by the calculated MPNs for the initial situation (spring 1988). Multiplication after two beet crops was estimated by dividing MPNs of 1990 by MPNs of 1988, both for BNYVV and *P. betae*. For 1989, multiplication ratios were calculated by dividing MPNs determined in the spring of 1990 by those determined in 1989. The viruliferous proportion of the population was estimated in 1990, by dividing MPNs determined for BNYVV by MPNs for *P. betae*.

Statistical analysis. Statistical computations were made using GENSTAT 5 (Payne et al., 1988). The analysis was based on the split-plot design. Prior to analysis, angular transformation was performed on percentages of infected bait plants in the bioassay on soil samples from October 1988, in order to improve homogeneity of error variances (Mead and Curnow, 1983). Analysis of variance (ANOVA) was performed on log-transformed MPNs ($\log_{10}$(MPN+1)), multiplication ratios ($\log_{10}$) and percentage viruliferous *P. betae* ($\log_{10}$(%+1)), because of the increase in variance with an increase in magnitude of the untransformed data. Means of log values were back transformed and presented as geometric means of the original data (Steel and Torrie, 1980).

Orthogonal polynomial contrasts were used to examine linear and quadratic trends in data. Although not meant to be used for responses to a quantitative factor (Baker, 1980; Dawkins, 1981), a LSD was given and Duncan's new multiple range test was applied (Duncan, 1955) for discriminating between means, only when interaction between factors was absent. This was done to see whether the (quantitative) bioassay methods used could distinguish between the plots with different initial inoculum levels, after growing one or two beet crops. As no inoculum had been applied to the control plots, they were omitted from the analysis of the multiplication ratio.

The log-transformed MPNs for BNYVV determined in 1990 and the multiplication ratio in 1989 were regressed against the log-transformed MPNs in 1989. Two plots in which BNYVV was not detected in 1989 were excluded from the analysis. Stepwise regression was performed with pooled data of non-irrigated and irrigated plots. In order to consider the split-plot design in the regression, first the factors block and block times irrigation (representing non-irrigated and irrigated main plots or strips) were fitted. Then the factors irrigation, $\log_{10}$ MPN and their interaction were added to the model. The significance of the regression on $\log_{10}$ MPN was determined with F tests. The significance of the irrigation effect was tested by comparison of fitted lines for each strip. The percentage of

variance accounted for by the regression in the interactive model was calculated considering the split-plot structure and is presented as a partial R^2 adjusted. To justify the use of $\log_{10}$ MPN 1989 for explanation of parameters measured in 1990, the absence of significant contributions of the factor 'initial inoculum level' and its interaction with irrigation to the regression model were checked. Fit of the linear model was assessed by examination of the significance of the regression, the percentage of variance accounted for and the plots of standardized residuals versus expected values for the regressions. Non-linear models, polynomial with a quadratic term and exponential, were fitted to the data and results compared with those of the linear model.

The correlation between (angular transformed) percentages of BNYVV-infected plants in the bioassay of October 1988 and the MPN of spring 1989 was determined.

Results

Field observations. In 1988, the beet crop developed well, only a slight yellowing of the leaves was observed in plots of the highest inoculum level and low numbers of BNYVV-infected plants were detected. In 1989, sowing was late and emergence was irregular because of drought. Plants with root symptoms of rhizomania were observed from the end of June onwards. Disease incidence was high.

Soil temperatures above 15 °C were recorded soon after sowing in both years. The frequency of rainfall events (≥ 1 mm per day, or occasionally per 2 days) and of irrigations after emergence of the plants till harvest was, respectively, 52 (421 mm accumulated) and 11 in 1988, and 37 (292 mm accumulated) and 7 in 1989. In 1988, long dry periods did not occur and the soil moisture tension did not attain extreme values. Irrigation kept the soil moisture tension below 20–30 cbar at 15 cm depth at times when it would have exceeded these values without irrigation. In 1989, there were three very dry periods and, since timing and execution of irrigation were not always as recommended, also the irrigated plots were from time to time subjected to high soil moisture tensions.

Detection of BNYVV in soil in 1988. In the experimental field, BNYVV could not be detected in samples taken before the start of the trial. After application of the infested soil to the field, the inoculum could only be traced in soil samples from two plots of inoculum level 3 and two plots of level 4 (Table 2). Immediately after harvesting the first beet crop, BNYVV was detected by bioassay at all inoculum levels. The average percentage of infected bait plants increased with increasing inoculum levels. Irrigation significantly increased these percentages (no significant interaction between inoculum level and irrigation). Three levels of infestation were distinguished by means of this bioassay (Table 2).

As at this time the most probable number method for estimation of infestation with rhizomania had not yet been developed, no quantitative assessments of BNYVV were made.

Quantitative assessment of BNYVV in soil in 1989 and 1990. For a quantitative assessment of the inoculum potential after growing one beet crop, soil samples were taken in spring 1989. Two plots in which no virus was detected in October 1988 (at inoculum level 0 and 2), were BNYVV-positive in May 1989, for one plot (inoculum level 0) the situation was the reverse. The MPN determined in May 1989 showed a positive correlation with the percentage of infected bait plants in the bioassay of October 1988, either trans-

Table 2. Detection of BNYVV by bioassay in soil samples from five inoculum levels, taken at the beginning and the end of the growing season in 1988. Six plots per inoculum level: three non-irrigated (NI) and three irrigated (IR). One sample per plot, ten plants per sample. Data are numbers of BNYVV-positive plots and arithmetic means of percentages and angular transformed percentages of BNYVV-infected plants.

Inoculum level[a] or irrigation	Number of BNYVV-positive plots		Means of percentages of BNYVV-infected bait plants				
			April[b]		October		October
	April[b]	October	NI	IR[c]	NI	IR	Mean angular[d]
0	–	2	–	–	0	7	6 a
1	0	4	0	0	3	40	24 ab
2	0	5	0	0	7	54	30 b
3	2	6	13	0	67	93	69 c
4	2	6	13	0	83	100	83 c
Significance (P-value)[e]							<0.001
NI							30
IR							55
Significance (P-value)[e]							0.03

[a] For explanation see Table 1.
[b] Due to an incident in the greenhouse all bioassay plants of level 0 (–), and 10–40% of those from levels 1, 2 and 4 were lost.
[c] Irrigation had not yet been applied prior to this sampling date.
[d] Mean values for the levels of each factor are given, because interaction between factors was not significant. Means with the same letter are not significantly different according to Duncan's new multiple range test ($P = 0.05$). LSD (0.05) = 21.
[e] Significance = F probability of main effects in ANOVA of angular transformed percentages. Interaction between irrigation and inoculum level was not significant at P=0.05. Analysis of orthogonal polynomial contrasts showed a significant linear (P<0.001), but no quadratic effect of initial inoculum level.

formed or untransformed (R^2 adjusted = 74%, $P < 0.01$).

Table 3 shows the geometric mean inoculum potential of BNYVV per inoculum level as determined in May 1989 and 1990. After one year, increasing MPNs were found with increasing initial inoculum level. In 1989, the MPNs were significantly different for the various initial inoculum levels. Irrigation in 1988 significantly influenced the MPN determined in the following year (Table 3, Fig. 2). Significant differences between plots that had different initial inoculum levels were also recorded in 1990, and two years of irrigation still resulted in higher MPNs as compared to the non-irrigated plots (Table 3, Fig. 2). The size of the effect of one or two years of irrigation did not depend on the initial inoculum level in either year; apparent interaction of inoculum level and irrigation was not significant. One of the irrigated control plots showed a severe contamination of the front part of the plot in 1990, therefore it was excluded from the calculations.

The effect of two years of extra moist conditions on the inoculum potential in 1990, as demonstrated by the analysis of variance, could be the result of the effect of irrigation in 1988 only or in combination with an effect in 1989; therefore regression analysis was carried out. The linear model gave the best fit for the range of values available (Fig. 3). The

Table 3. Inoculum potential of BNYVV in soil after growing one or two beet crops in an artificially infested field (May 1989 and May 1990). Assessment of the most probable numbers (MPN) of infective units by means of the soil dilution method, dilution ratio 10, six replicates per dilution. Data are geometric means of three MPNs per inoculum level and arithmetic means of log-transformed MPNs. *P*-values refer to main effects in ANOVA.

Inoculum level or irrigation[a]	May 1989			May 1990		
	MPN BNYVV/100 g soil[b]		Mean $\log_{10}$ MPN BNYVV	MPN BNYVV/100 g soil[b]		Mean $\log_{10}$ MPN BNYVV
	Non-irrigated	Irrigated		Non-irrigated	Irrigated	
0	0.2	0.3	0.10 a[c]	15	150	1.69 a[c]
1	0.6	7	0.53 b	32	450	2.09 a
2	12	44	1.38 c	200	840	2.61 b
3	80	280	2.17 d	340	1200	2.81 b
4	630	1100	2.91 e	1700	3700	3.40 c
Significance (*P*-value)[d]			<0.001			<0.001
NI			1.21			2.56
IR			1.63			2.97
Significance (*P*-value)[d]			<0.01			0.04

[a] For explanation see Table 1. NI = non-irrigated, IR = irrigated.
[b] Geometric means: back transformed mean $\log_{10}$ MPNs. For plots in which BNYVV was not detected (four and two respectively, for inoculum levels 0 and 1 in 1989) calculations were made using MPN = 0.
[c] Means with the same letter are not significantly different according to Duncan's new multiple range test ($P = 0.05$). LSD (0.05) was 0.33 for 1989 and 0.46 for 1990.
[d] Significance = *F* probability of main effects in ANOVA of $\log_{10}$ (MPN+1). Interaction between irrigation and inoculum level was not significant at $P = 0.05$ in either year. Analysis of orthogonal polynomial contrasts showed a significant linear ($P < 0.001$), but no quadratic effect of initial inoculum level.

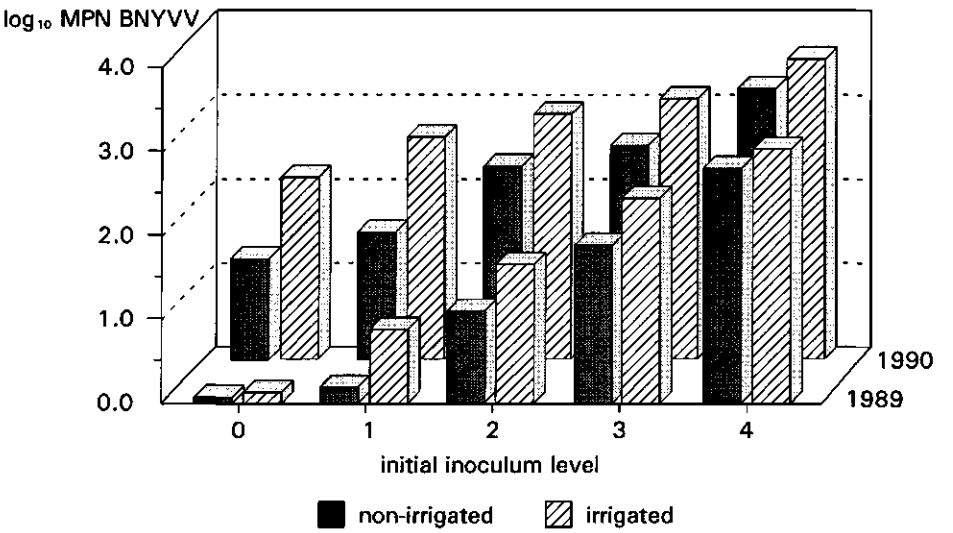

Fig. 2. The inoculum potential of BNYVV in soil in May 1989 and May 1990, after growing one or two beet crops, respectively, in an artificially infested field. Five inoculum levels, with or without irrigation. Inoculum potential given as arithmetic means of $\log_{10}$(MPN + 1); MPN is the most probable number of infective units per 100 g soil.

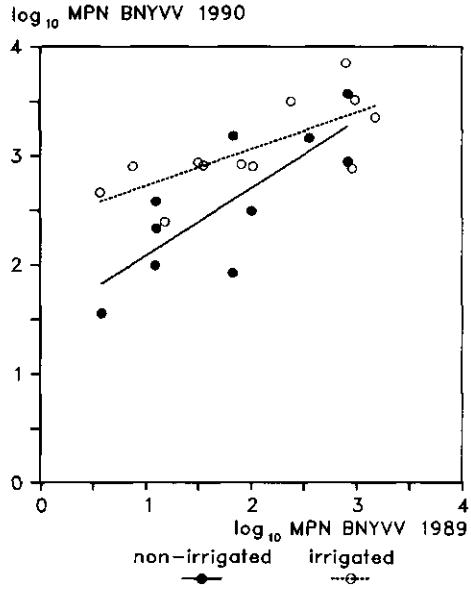

Fig. 3. Relationship between the inoculum potentials of BNYVV in 1990 (Y) and in 1989 (X). Regression was based on individual assessments of all initially infested plots, except for two plots that had an inoculum level in 1989 below the detection level of the bioassay (X = 0.20). Analysis of $\log_{10}$ (Most Probable Number/100 g soil + 1). Regression was significant ($P < 0.001$) with partial R^2 adjusted = 73%. The effect of irrigation was not significant at $P = 0.05$. Equations for the non-irrigated and irrigated data-set were, respectively: $Y = 1.46 + 0.62X$ and $Y = 2.38 + 0.34X$.

apparent effect of irrigation on individual MPNs was not statistically significant ($P = 0.05$) in an analysis of the effect with strips as experimental units because of the split-plot structure.

Multiplication ratio of BNYVV (viruliferous P. betae). For the severely infested soil that was used to contaminate the field in 1988 the MPN of BNYVV was 710 per 100 ml soil, the viruliferous population comprising 15% of the total *P. betae* population. Using this MPN, the volumes of infested soil added to the plots in 1988 and the bulk density of the field soil, the initial inoculum levels could be estimated as $6.6 \cdot 10^{-5}$ per 100 g soil for level 1 to 0.066 for level 4. Division of the MPNs assessed for 1989 by the ones calculated for 1988 indicated a ten-thousandfold multiplication of BNYVV in the first year (Table 4). The multiplication ratio decreased with increasing initial inoculum level and irrigation enhanced multiplication at all levels.

With the MPNs determined in 1990, the overall multiplication after two beet crops was assessed in the same way as that after one (Table 4). These two-year ratios also showed a decrease with increasing initial inoculum levels. The difference in two-year ratios due to two years of irrigation was not significant at $P = 0.05$.

Actual multiplication during the second year was estimated by dividing MPNs determined in 1990 by those of 1989. Geometric mean values for multiplication ratios ranged from 70 for the lowest inoculum level in irrigated plots to around 3 for the highest level either irrigated or non-irrigated (Table 4). Two years of irrigation had no effect on the multiplication ratio in the second year. Because irrigation had caused a significant increase of MPNs in the first year, initial inoculum levels in 1989 were not comparable. Thus, stepwise regression was performed to analyse effects in the second year (Fig. 4). Log_{10} transformed multiplication ratios decreased linearly with increasing $\log_{10}$ MPNs ($P < 0.001$). The apparent effect of irrigation was not significant ($P = 0.05$).

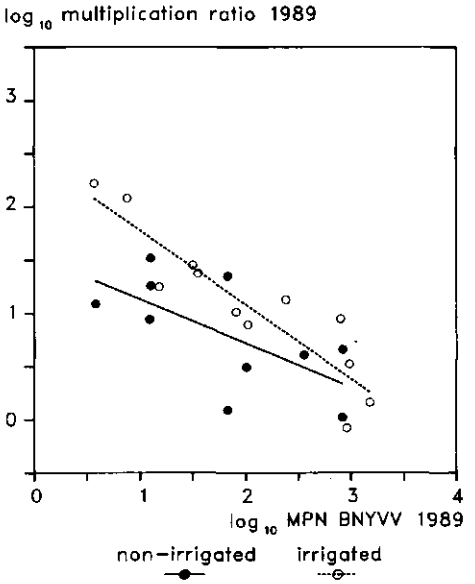

Fig. 4. Relationship between $\log_{10}$ multiplication ratio in 1989 (Y) and the inoculum potential of BNYVV in May 1989 (X). Regression was based on individual assessments of all initially infested plots, except for two plots that had an inoculum level in 1989 below the detection level of the bioassay ($X = 0.20$). Regression was significant ($P < 0.001$) with partial R^2 adjusted = 81%. The effect of irrigation was not significant at $P = 0.05$. Equations for the non-irrigated and irrigated data-set were, respectively: $Y = 1.55 - 0.41X$ and $Y = 2.47 - 0.70X$.

Quantitative assessment of P. betae. In the bioassays on soil samples taken in 1987 only 0, 1 or 2 bait plants were infected by *P. betae*. The low incidence of *P. betae* permitted an estimation of numbers of infective units of *P. betae* in soil by means of this 'dilution'. In 1987, inoculum potential of *P. betae* in the field prior to infestation was 0.19 infective units per 100 ml soil or 0.14 per 100 g soil. For the infested soil applied to the field in 1988 the MPN of *P. betae* was 4800 per 100 ml soil. By means of this MPN and the volumes of soil applied to the plots, the numbers of infective units added to the resident population were calculated. In this way, the initial inoculum levels could be estimated; they ranged from 0.14 to 0.58 infective units of *P. betae* per 100 g soil for level 0 and level 4, respectively. Because of the presence of the resident population, the initial levels of *P. betae* did not differ by a factor of 10. The initial levels of *P. betae* for the inoculum levels of BNYVV from 0 to 4 given in proportion to that at BNYVV level 4 were 0.25, 0.25, 0.25, 0.32 and 1.0.

Bait plants of the quantitative bioassays in 1990 were examined for the presence of *P. betae* (Fig. 5). The MPNs were high and only that for inoculum level 4 was significantly different from the rest (Table 5). Two years of irrigation had caused an overall increase of the population. Two-year multiplication ratios were calculated using the MPNs in 1990 and the estimated MPNs in 1988. Multiplication was the same for all inoculum levels (ANOVA of $\log_{10}$ multiplication ratio, $P = 0.05$), but had been enhanced by irrigation ($P < 0.01$), as was already apparent from the MPNs themselves. Mean $\log_{10}$ transformed multiplication ratios were 4.3 and 4.8 for non-irrigated and irrigated plots, respectively; back transformed means were 2.2×10^4 and 6.3×10^4.

The viruliferous part of the population of *P. betae* was estimated by dividing the MPN of the virus by that of the vector. The initial viruliferous percentages in 1988 were estimated as 0, 0.04, 0.4, 3.4 and 11.0% of the total population for inoculum levels 0 to 4, respectively. By 1990 they had increased to 1, 2, 8, 12 and 12% of the total population (Table 5).

Table 4. Estimated multiplication ratios of BNYVV (viruliferous *Polymyxa betae*) at different initial inoculum levels, calculated after growing the first (1988) and the second (1989) beet crop. Data are geometric means of multiplication ratios and arithmetic means of log-transformed multiplication ratios. *P*-values refer to main effects in ANOVA.

Level[a]	One beet crop (1988)			Two beet crops (1988 + 1989)			The second beet crop (1989)		
	Multiplication ratio (x10000)[b]		Mean $\log_{10}$ ratio	Multiplication ratio (x10000)[b]		Mean $\log_{10}$ ratio	Multiplication ratio		Mean $\log_{10}$ ratio
	NI	IR		NI	IR		NI	IR	
1	4[c]	10	4.8	50	740	6.3	33[c]	71	1.7
2	2	7	4.6	31	130	5.8	17	19	1.3
3	1	4	4.4	5	19	5.0	4.4	4.4	0.7
4	1	2	4.1	3	6	4.6	2.7	3.5	0.5
Significance (*P*-value)[d]			0.01			<0.001			<0.01
NI			4.2			5.1			1.0
IR			4.7			5.8			1.1
Significance (*P*-value)[d]			<0.01			0.08			0.71

[a] Inoculum level; for explanation see Table 1. NI = non-irrigated, IR = irrigated.
[b] Values presented should be multiplied by 10 000.
[c] For two plots of this treatment BNYVV was not detected in 1989, whereby multiplication ratios could not be determined. Calculations were made using missing values estimated by an iterative approach in GENSTAT5.
[d] Significance = *F* probability of main effects in ANOVA of log-transformed multiplication ratios. Interaction between irrigation and inoculum level was not significant at $P = 0.05$ in either year. Analysis of orthogonal polynomial contrasts showed a significant linear effect in both years ($P < 0.01$, $P < 0.001$ and $P < 0.01$ for 1988, 1988+1989 and 1989, respectively), but no quadratic effects of initial inoculum level. LSD (0.05) was 0.38 for 1988, 0.42 for 1988+1989 and 0.61 for 1989.

Discussion

Qualitative detection of BNYVV in 1988. BNYVV was not detected prior to the introduction of infested soil to the experimental field. The different amounts of BNYVV-infested soil added to the plots created initial inoculum levels that could hardly be traced by intensive soil sampling followed by bioassay. It may be assumed that the infective units applied to the plots were randomly dispersed and that samples obtained by means of the regular sampling grid were representative of the plots. Thus, comparison of the detection limit of the bioassay used (0.08 infective units per 100 g soil) with the highest initial inoculum level (approximately 0.06 infective units per 100 g soil) explains why BNYVV was only detected in a few plots.

Growing a susceptible beet crop resulted in a remarkable increase in the inoculum levels after only one year. Even at the lowest initial inoculum levels plant roots encounter

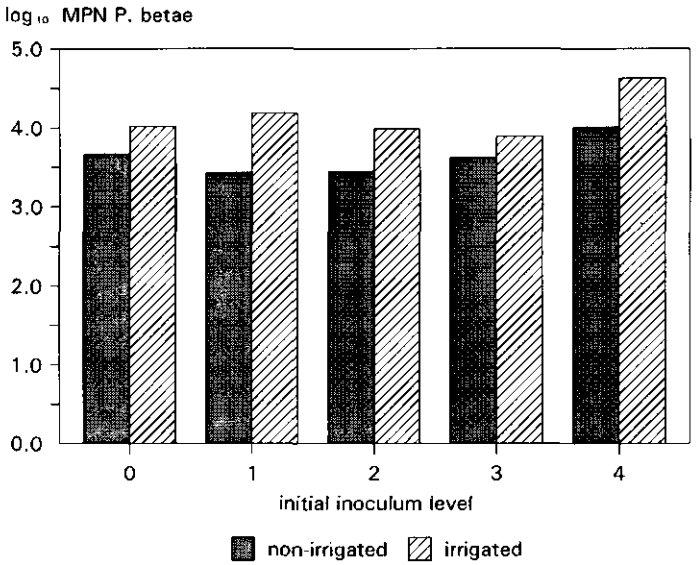

Fig. 5. The inoculum potential of *Polymyxa betae* in soil in May 1990, after growing two successive beet crops in an artificially infested field. Five inoculum levels, with or without irrigation. Inoculum potential presented as $\log_{10}$(MPN + 1); MPN is the most probable number of infective units per 100 g soil.

infective propagules, because during the season the root mass extends and root density increases, so that a large area will be explored by roots, to varying depths and resulting in various root densities (Brown and Biscoe, 1985). The total number of plants that became infected and the time of primary infection of plants differed for the different low inoculum levels of 1988, resulting in different amounts of infected root tissue. Soil samples taken in October of 1988 and analysed by bioassay showed the presence of BNYVV at all inoculum levels. Apparently, an abundance of resting spores formed during the season was already able to germinate, in the bioassay, immediately after harvest of the crop and did not need a long maturation period after being liberated from infected root debris. The average percentage of infected bait plants increased with increasing inoculum level, indicating that differences between these levels were still present and could be detected even though the initial random pattern of propagules had probably changed to randomly dispersed clusters of propagules.

In spite of phytosanitary precautions taken, after one year three of the control plots had become contaminated; two of these contaminations were detected in October of the first year, the additional one in May of the second year. The contamination of the control plots could have been caused by a number of factors, including dispersal by wind (at the time of application of infested soil), water, animals and man.

Quantification of BNYVV in 1989 and 1990. Soil samples taken after winter should be more representative of the plots as a whole than those taken at an earlier time after harvest. Cultivation of the soil should have distributed the root remnants more evenly through the

Table 5. Analysis of the inoculum potential of *Polymyxa betae* in soil and of the percentage of the viruliferous population in May 1990, after two successive beet crops in an artificially infested field. Assessment of the most probable numbers (MPN) with the soil dilution method, dilution ratio 10, six replicates per dilution. ANOVA of $\log_{10}$(MPN + 1) and $\log_{10}$ (% viruliferous population + 1). Data are geometric means of MPNs and percentages and arithmetic means of their log-transformed values. *P*-values refer to main effects in ANOVA.

Inoculum level or irrigation[a]	Inoculum potential of *P. betae*		Viruliferous *P. betae* population	
	MPN/100 g	$\log_{10}$(MPN+1)	%	$\log_{10}$(% virul.+1)
0	7000	3.85 a[b]	1	0.34 a[b]
1	6500	3.81 a	2	0.49 a
2	5200	3.72 a	8	0.95 b
3	5700	3.76 a	12	1.10 b
4	20700	4.32 b	12	1.13 b
Significance (*P*-value)[c]		<0.01		<0.001
NI		3.64		0.73
IR		4.14		0.87
Significance (*P*-value)[c]		<0.01		0.29

[a] Inoculum level; for explanation see Table 1. NI = non-irrigated, IR = irrigated.
[b] Means with the same letter are not significantly different according to Duncan's new multiple range test ($P = 0.05$). LSD (0.05) for log (MPN + 1) was 0.28 and for log (% viruliferous+1) 0.33.
[c] Significance (*P*-value) = *F* probability of main effects in ANOVA. Interaction between irrigation and inoculum level was not significant at $P = 0.05$ for either parameter. Analysis of orthogonal polynomial contrasts showed a significant linear ($P < 0.01$) and quadratic ($P < 0.01$) effect of initial inoculum level on $\log_{10}$(MPN + 1). For $\log_{10}$(percentage viruliferous population + 1) only a linear effect was significant ($P < 0.001$).

tillage layer, roots should be more disintegrated and resting spores may have matured. Therefore, in spring 1989 soil samples were taken for quantitative assessment of the inoculum. In the only inoculated plot, of inoculum level 2, where BNYVV was not detected in October 1988, it was detected in the following spring. In two plots of level 1, non-irrigated, BNYVV was detected neither in spring 1989 nor in October 1988. Here, the mean inoculum level was still below the detection level of the quantitative bioassay (calculated as MPN = 0.6 infective units per 100 g soil). Assuming that all infective units introduced in 1988 resulted in clusters of infective propagules with a radius of 30 cm, based on spread from an inoculum source (Tuitert, 1993) the (Poisson) probability of obtaining one infested soil core would be approximately 0.05. Although soil cultivation in autumn 1988 and spring 1989 would enlarge but also dilute the infested patches, the chance of missing infested areas during the sampling procedure cannot be disregarded.

MPNs of BNYVV determined at successive inoculum levels in 1989 did not differ by a factor of 10, but differences between the initially applied densities were reflected (Table 3). For *Phytophthora parasitica*, another fungus with a secondary multiplication cycle, Neher and Duniway (1991) also found that population densities during the season ranked according to volumes of inoculum added to the soil. Mean log MPNs enabled a distinc-

tion between the five original inoculum levels (Table 3). The bioassay results of October 1988 permitted a division into three groups of infestation levels only (Table 2), despite the significant correlation of the (angular transformed) bioassay results with log MPNs in 1989. It is noticeable that after one year mean MPNs of inoculum level 4 attained values corresponding to the MPN of the soil used as inoculum in 1988.

In 1990, MPNs had increased as compared to 1989. The significance of main effects in ANOVA showed that after two successive beet crops inoculum levels were still determined by the initial levels of inoculum applied and that two years of irrigation resulted in higher MPNs. The MPNs increased with increasing initial inoculum level, and three groups of levels could be distinguished in 1990 (Table 3). In all control plots contamination had increased to detectable levels. Because irrigation had caused an increase in inoculum in the first year, the initial levels in 1989 for non-irrigated and irrigated plots were no longer identical. By regression analysis a significant linear relation was shown between log-transformed MPNs of both years.

The increase in BNYVV per year. The estimated ten-thousandfold increase in inoculum levels of BNYVV during 1988 reflects the high reproductive potential of *P. betae* by means of its secondary cycle. Multiple infection of plants might have been responsible for the observed decrease in multiplication ratio with increasing inoculum level in the first year. Multiplication ratios in 1989 were small compared to those in 1988; mean ratios for low to high inoculum levels ranged from approximately 70 to 3.

Assessments of changes in the population of *P. betae* and/or BNYVV in soil have not been made before, only reports on the colonization of roots by *P. betae* are available. In the early work of Kanzawa (1974) a linear correlation was found between the numbers of resting spore clusters (seven concentrations each differing by a factor of 10) added to the soil and the percentage of rootlets infected by *P. betae* after 40 days. Bürcky et al. (1986) reported an increase in the number of *P. betae*-infected root pieces and intensity of infection with resting spores with increasing inoculum level (0.1–100% dilutions of infested soil). Unfortunately, their assessments were neither really quantitative nor statistically tested. With values read from figure 3 of Goffart et al. (1987), a logistic increase of mean numbers of resting spores of *P. betae* per g root of bait plants with increasing concentration of spores in the soil can be demonstrated. The estimated multiplication ratio of the fungus in the 5-week period was lowest at the highest density of spores in soil (dilutions of infested soil tested: 0.006–100%). Since the amount of spores was given per g of root and total root weights of plants at the different soil dilutions were not presented, these calculations cannot be translated to soil populations.

Rintelen and Walla (1985), on the other hand, did not detect differences in percentages of infected root pieces in roots of sugar beet grown for 5 weeks in dilutions of infested soil (1–33%). These assessments did not take into account the degree of infection of the root pieces. Very likely, differences were not observed because the soil used had a high inoculum level and the dilution range was too limited.

Results may be different when *P. betae* is examined, which is the case with the roots, as compared to BNYVV only, done in our experiment. Nevertheless, the conclusion of Rintelen and Walla (1985) that the amount of infection in the roots is not dependent on the degree of contamination of the soil appears to be contradicted by the results presented here.

Decreasing multiplication ratio of BNYVV in 1989. Regression analysis revealed that log-transformed multiplication ratios decreased linearly with increasing log MPN. The reason for the lower multiplication at higher inoculum levels may be found in the availability of infection sites. At high levels of inoculum, sites on roots will quickly be occupied through primary infection, so sites for secondary infection will sooner become limiting at these levels compared to lower inoculum levels, where primary infection is less frequent and larger zoosporangia might be formed (Keskin, 1964).

There is no reason to suppose that there would be a decrease in the proportion of resting spores germinating at higher inoculum levels. But it is not known what proportion of a population will germinate during the season, and if this proportion is influenced by virus content of the resting spores. A complicating factor with *P. betae* is that perhaps only a fraction of the resting spores contained in one cluster will germinate (Habibi, 1969); so the germinated 'cluster' continues to be an infective unit.

The trigger in the infection process determining if a plasmodium follows the pathway to zoosporangium or resting spore is unknown. At some stage there may be a general change from zoospore production to the formation of resting spores (Asher and Blunt, 1987). Possibly the onset and impact of that trigger is influenced during the infection process, e.g. by the abundance of initial infection.

An interacting factor could be the influence of the transmitted virus on root growth. BNYVV may inhibit root growth and cause roots to die. To compensate for the loss of roots and the effects of blocked vessels, plants will form extra roots; a proliferation well-known as a rhizomania symptom. The net effect of these phenomena on multiplication of *P. betae* is not known. Non-viruliferous *P. betae* can acquire BNYVV from systemically infected roots (Abe and Tamada, 1986). There are, however, records of inhibition of infection of roots regenerated from BNYVV-infected tap roots by non-viruliferous *P. betae* (Schlösser, 1990). The efficiency of acquisition of virus from infected roots by *P. betae* will of course play a role in the increase in BNYVV. Alteration of epidemic components of a fungal (leaf) disease by simultaneous virus infection of the plant has been observed by Nelson and Campbell (1991).

The observation of decreasing multiplication with increasing inoculum level indicates that when effects of different factors (e.g. resistant cultivars) on BNYVV in soil are studied, multiplication ratios may not be directly comparable unless initial inoculum is more or less uniform, or a range of inoculum levels is used. Effects of sanitation (the reduction of initial inoculum, c.f. Van der Plank, 1963), on the inoculum levels in soil are likely to be quickly diminished when a beet crop is grown, because of the accompanying increase in multiplication of BNYVV at lower inoculum levels.

Two-year multiplication ratios of BNYVV and P. betae. Two consecutive beet crops resulted in a higher multiplication ratio for BNYVV at the lowest inoculum levels. The *P. betae* population already present at the experimental site was high relative to the small amounts of inoculum added in 1988. Only the *P. betae* population at inoculum level 4 was increased (threefold) by the addition of inoculum. Two-year multiplication ratios for *P. betae* were equal for all levels, thus resulting in similar MPNs for levels 0 to 3, and a higher MPN at level 4. Apparently, the initial differences in virus content of the *P. betae* populations did not affect the multiplication of *P. betae*. The higher two-year multiplication ratio for BNYVV than for *P. betae* at low inoculum levels (even when BNYVV levels

are corrected for the MPNs found at the uninfested control) is striking. It should be emphasized that initial inoculum levels for BNYVV each differed by a factor of 10, but that this was not the case for *P. betae*, because of the presence of the resident population. This circumstance interferes with the comparison of multiplication ratios for vector and virus at equal initial inoculum levels of BNYVV. Even so, it appears that virus acquisition by *P. betae* was more efficient at low initial inoculum levels. Once BNYVV has multiplied in a root, at low inoculum levels a majority of non-viruliferous zoospores infecting the root will acquire the virus during plasmodium formation. At high inoculum levels, many zoospores infecting the BNYVV-infected root will already be viruliferous, and thus the net increase in viruliferous propagules will be less. Furthermore, there may be more secondary infection cycles at the lower inoculum levels, causing an increasing virus content and spread in the roots, compared to the high inoculum levels, where primary infection will be more prominent.

By dividing the MPNs of BNYVV and *P. betae* an estimation of the viruliferous proportion of the population of *P. betae* can be obtained, on the assumption that the rate of germination and infection is the same for viruliferous and non-viruliferous *P. betae* (Tuitert, 1990). Implicitly it is also assumed that for successful infection of bait plants by *P. betae* or BNYVV the same number of non-viruliferous or viruliferous propagules is required. Because of these assumptions the real viruliferous percentage of the population may be over- or underestimated; however, the figure enables comparison of different soil samples. The geometric mean viruliferous percentages ranged from 1 to 12%. At the three highest inoculum levels the viruliferous percentage of the population of *P. betae* after two beet crops corresponded with the percentage of that in the soil used as inoculum in 1988. In comparison, 1–2% of secondary zoospores of an efficient viruliferous isolate of *P. graminis* appeared to be carrying particles of barley mild mosaic virus (Jianping et al., 1991).

Influence of soil moisture conditions. Drip irrigation enhanced the increase in MPN of BNYVV in both years (Table 3). The *P. betae* population was also higher following two irrigated crops compared to the non-irrigated situation (Table 5). Irrigation may have stimulated multiplication by the formation of extra primary zoospores, by extra secondary cycles, and both possibly combined with a greater 'efficiency' of infection by the zoospores released (e.g. by swimming further). All these effects would result in a multiplicative effect compared to the non-irrigated treatments, which is additive when transformed to log values. Since irrigation may cause an increase in root density, especially in the tillage layer (Brown et al., 1987; Wild and Russell, 1988), extra availability of infection sites may be an important factor in the increase in inoculum caused by irrigation, especially at already high initial inoculum levels.

Soil moisture conditions in 1988 appeared to have favoured fungal development. In June a dry period may have inhibited the life cycle of *P. betae*, and irrigation may then have exerted its influence on multiplication. Irrigation was responsible for a threefold higher multiplication ratio of BNYVV in 1988 (Table 4; antilog value of 4.7 minus 4.2). The objective of maintaining a threshold soil moisture tension of approximately 20 cbar at 15 cm depth in the irrigated plots was not always achieved. De Heij (1991) reported that even lower tensions (10–12 cbar) were required in pot experiments for primary and secondary infection to occur. It is difficult to translate these precisely measured threshold tensions from pot experiments using compact sieved soil to field conditions where soil is not

homogeneous and where tensions are measured less precisely. Soil moisture tensions at depths greater than 15 cm were lower but, especially in the first year, infection by the superficially raked-in inoculum will mostly have taken place in the upper soil layers. Preliminary results of Gerik et al. (1990) indicated that infection could take place at tensions up to 40 cbar. For *Plasmodiophora brassicae*, germination of resting spores and primary infection was recorded at tensions up to 20 cbar (Dobson et al., 1982). Soil moisture requirements for zoospore movement will also depend on the type (texture) of soil (Duniway, 1976; Dobson et al., 1982).

In 1989, there were three very dry periods and from time to time high tensions were recorded at 15 cm depth, and to a lesser extent at 30 cm depth. Nevertheless, differences in soil moisture conditions between irrigated and non-irrigated plots were less conspicuous than in 1988. Regression analysis revealed that irrigation in the second year had no significant effect on log MPN BNYVV in addition to irrigation in the first year.

The combined effect of two years of irrigation on log MPNs of BNYVV was just significant (Table 3). Both one and two years of irrigation caused an approximately threefold increase of MPNs of BNYVV (Table 3; antilog values of 0.42 and 0.41, respectively). Because higher MPNs caused by irrigation in the first year have lower multiplication ratios, a possible increase in MPNs by irrigation in the second year would be partly hidden by this reverse effect of inoculum level. The apparent smaller increase by irrigation at high inoculum levels (Fig. 2), probably approaching 'saturation' level, was not significant because interaction of inoculum level and irrigation was not significant. When inoculum level 0 was excluded from the analysis of MPNs in 1990, the effect of irrigation was not large enough to be detected significantly (P=0.08). The effect of irrigation on the two-year multiplication ratios of BNYVV was therefore also not significant at the confidence level applied (Table 4). Two-year multiplication ratios of *P. betae* were significantly increased by two years under irrigated conditions.

Conclusion. The research described in this paper is the first report on the population dynamics of viruliferous *P. betae*. The results indicate the potential hazards of introduction of small amounts of rhizomania-infested soil to a field. With an evenly distributed infestation and favourable conditions for development of *P. betae* and BNYVV the build-up of inoculum can be very fast.

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Chapter 7

Horizontal spread of beet necrotic yellow vein virus in soil

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Horizontal spread of beet necrotic yellow vein virus in soil

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Abstract

Horizontal dispersal of beet necrotic yellow vein virus (BNYVV) by means of viruliferous zoospores of *Polymyxa betae* was studied in greenhouse experiments. BNYVV was not detected in roots of sugar beet plants grown in silver sand for 4 weeks at a root-free distance of 5 cm from either *P. betae*- and BNYVV-infected plants or BNYVV-infested soil. Spread of BNYVV from inoculum sources in the field was studied in the absence and presence of tillage practices. Active dispersal in combination with root growth from and towards point sources of inoculum contributed only little to horizontal dispersal of viruliferous inoculum and spread of disease during the season, as determined for one soil type, two different years and in the absence of tillage and tread. In the second beet crop after application of inoculum to whole field plots, more BNYVV-infected plants were detected at 2 m than at 8 m distance from the infested plots in the tillage direction. In the third year, disease incidence at 8 m was high and equivalent to that at 2 m.

Additional keywords: BNYVV, *Polymyxa betae*, epidemiology, dispersal, tillage

Introduction

Epidemiological studies on rhizomania of sugar beet require knowledge of distances of spread of the disease. The causal agent, beet necrotic yellow vein virus (BNYVV), is transmitted by the fungus *Polymyxa betae*. Spread of the virus thus mainly depends on the behaviour of the vector and on root development of the plant. By definition a pathogen spreads where it goes and infects, and dispersal is the movement of propagules from infected tissue or plants to healthy susceptible tissue or plants (Van der Plank, 1967). Different modes of dispersal of viruliferous propagules of *P. betae* through or with soil can be distinguished. First, there is 'active dispersal' by zoospores moving autonomously through soil. Second, there are various ways in which propagules of *P. betae* are 'passively dispersed', i.e. without having to spend their energy. Zoospores can be passively displaced by water currents over the soil surface or through soil pores. The persistent resting spores can be dispersed by means of movement of contaminated soil and manure and by water (Hillmann, 1984; Heijbroek, 1987).

In a greenhouse experiment, distances covered by active dispersal were investigated. Field trials were set up in 1988 and 1989 in order to estimate distances of spread of rhizomania from point sources of inoculum in a sugar beet crop under field conditions, in the absence of soil tillage. In addition, in plots of the 1988 trial, it was studied how the extension of the different original inoculum sources manifested itself in a second beet crop. Spread of rhizomania by displacement of infested soil was investigated in another field trial.

Material and methods

I. Experiments with inoculum sources

Greenhouse experiment

Dispersal of viruliferous zoospores was assessed by growing target plants at various root-free distances from different inoculum sources. In wide shallow trays, 30 × 30 × 8 cm, 2 cm wide and 30 cm long pockets of Monodur gauze (50 μm) were placed, filled with either a mixture (50/50) of rhizomania-infested soil and silver sand or silver sand only and planted with sugar beet seedlings cv. Regina infected by a short incubation in infested soil (according to Beemster and De Heij, 1987). Trays were filled with sterilized fine silver sand to which 0.25 g NPK (14–16–18) and 0.30 g lime fertilizer per l were added, the pH was thus adjusted to 7.0. Target plants, sugar beet cv. Regina, were sown at distances of 5 and 10 cm from the two types of inoculum sources. Their root growth towards the inoculum sources was prevented by Monodur gauze (50 μm). Two controls were included: trays without an inoculum source and trays of which the upper layer of 1–2 cm was infested with rhizomania-infested soil. Each treatment was replicated three times. Water was given regularly from below (soil water potential fluctuated between approximately –3 and –6 cbar). Temperature in the greenhouse was about 23 °C during daytime (16 h) and 15 °C at night. Two and four weeks after emergence (emergence about 6 days after sowing), the target plants were sampled and tested by ELISA for the presence of BNYVV, either directly or after 5 weeks growing in sterile silver sand.

Field experiments

In 1988 and 1989, two identical field trials were laid out on a calcareous clay soil at an isolated site in an urban area in the Noordoostpolder, the Netherlands. The trials were situated at adjacent parts of the field. Experimental details are given in Table 1. Sugar beet had never been grown in this field. Circular inoculum sources (described below) were created, target plants were grown at different distances from the sources and the area between target and source was devoid of plants (Fig. 1). Control plots were either non-in-

Table 1. Experimental details of the field trials in 1988 and 1989 on adjacent parts of a field.

Parameter	1988	1989
Soil		
pH-KCl	7.1	7.1
organic matter %	6.7	6.7
Layout		
plot size (m^2)	3 × 4	3 × 3
Sowing		
date	15 April	2 May
distance within row (cm)	5[a]	10
distance between rows (cm)	12.5	25
number of target plants at 25 cm, per plot	12	6
number of target plants at 50 cm, per plot	24	10
Inoculum		
MPNs[b] of infective units BNYVV	500	6

[a] Plants were thinned to a distance of approximately 10 cm.

[b] Most probable numbers per 100 g dry soil.

Fig. 1. Inoculum source in a field trial with target plants at a distance of 25 cm.

fested or infested by application of infested soil to the top layer. The five treatments (Table 2) with four replications were laid out in a randomized block design. Plots were separated by 3 m wide grass borders. In the available free space at the experimental location some extra plots with inoculum sources were arranged as described in Table 3.

Table 2. Treatments in the field trials of 1988 and 1989 and details of plant sampling at the end of the growing season.

Treatment	Type of inoculum source[a]	Approximate distance of target plants[b]	Number of target plants sampled			
			1988		1989	
			25 cm	50 cm	25 cm	50 cm
1	Soil without plants	25 cm	16	–	24	–
2	Soil with plants	25 cm	16	–	24	–
3	Soil with plants	50 cm	–	24	–	24
4[c]	Non-infested	–	8	12	12	12
5[c]	Superficially infested	–	8	12	12	12

[a] The circular inoculum sources in Treatments 1, 2 and 3 had a diameter of 20 cm and a depth of 12 cm. In Treatment 5, infested soil was spread evenly over the surface of the whole plot.
[b] Actual distances between target plants and inoculum sources varied between 20–30 cm and 45–55 cm for the distances presented as 25 and 50 cm.
[c] Plant samples were taken at positions corresponding to the sampling positions in Treatments 1–3.

Table 3. Treatments and assessments performed in single plots to determine BNYVV at different distances from inoculum sources, either in target plants by ELISA or in soil by bioassay.

Year	Treatment: inoculum source[a]	Assessments	BNYVV-infected plants		
			Target[b]	Bioassay[c]	
				20,25 cm	50,60 cm
1988	Circular source of soil in between two rows of beet; sowing distance 18.5 cm, 50 cm between the rows	All plants in three rows of 2 m length on either side of the source tested for BNYVV	0/59		
		Bioassay of soil samples at 20 and 60 cm distance		7/10	0/10
	Circular source of soil in fallow plot	Bioassay of soil sample at 20 cm distance		0/10	
1989	Circular source of soil in fallow plot	Bioassay of soil samples at 25 and 50 cm distance		0/10	0/10
	Circular source of soil with plants in a plot left fallow	Bioassay of soil samples at 25 and 50 cm distance		0/10	0/10

[a] Circular inoculum sources had a diameter of 20 cm and a depth of 12 cm.
[b] Distances between inoculum source and nine target plants in the adjoining row on either side of the source varied between 15 and 45 cm. The other 50 target plants grew at larger distances.
[c] Soil samples consisted of 40 cores, taken at the described distances. Bioassays were performed with 10 plants per soil sample, as indicated (/10).

Sowing. In both years sugar beet cv. Regina was sown. To ensure sufficient plants at the desired distances from the inoculum sources, sowing was dense (Table 1). The numbers of target plants per plot at a distance of 25 and 50 cm from the source are given in Table 1. Seedlings and weeds in the zone between inoculum source and target plants were removed directly after emergence.

Inoculum. Soil of the same rhizomania-infested field in the Noordoostpolder was used as inoculum in both years. However, quantitative assessments of the inoculum (Tuitert, 1990) showed a big difference in level of infestation between the two years (Table 1). Inoculum was applied within one week after sowing. In order to create the inoculum sources (Table 2, Treatments 1–3) a round hole was dug in the middle of the plot, with a diameter of 20 cm and a depth of 12 cm. The hole was carefully filled with a mixture of 2 l infested soil and 1 l non-infested soil from the trial field. The soil mixture was compressed to enable addition of a 2 cm thick top layer of non-infested soil. For Treatments 2 and 3 three seeds were sown in this site. Treatment 5 was infested with approximately 48 g soil per m^2 in 1988 and 3 kg soil per m^2 in 1989, equally spread over the top layer. The higher amount of soil applied in 1989 was based on a preliminary bioassay indicating a lower level of infestation of this soil. After the MPNs had been determined (Table 1), the amount used in the field appeared to be adequate, compared to 1988. To avoid spread by treading the plots, a movable bridge spanning the plots was used from which all activities were performed. Machinery was not used on the plots after inoculum had been applied.

Determination of BNYVV in target plants. Target plants at the nearest distances were sampled on 11 October 1988 and 15 September 1989 (Table 2). In 1988, plants were also periodically sampled during the season. From each plant, sap from the root tip was analysed by double antibody sandwich ELISA (Clark and Adams, 1977; Tuitert, 1990) for the presence of BNYVV. The plants growing in the sources were analysed too.

Determination of BNYVV in soil. Before establishing the trials, all plots were sampled to a depth of 25 cm with 42 cores of 1.3 cm diameter, taken in a regular grid (0.5 × 0.4–0.6 m). To register the extent of spread of BNYVV from the sources in soil, soil samples were taken in October 1988 and September 1989. After careful removal of the plants, preventing displacement of adhering soil, soil samples consisting of 40 cores were collected along a circle around the sources. For Treatments 1 and 2 sampling distances from the sources were 25 cm and 50 cm. In Treatment 3 the cores were taken at 50 cm in 1988 and at 25 and 50 cm in 1989. From the non-infested and infested control plots 40 cores were taken by sampling the whole area of the plots. Soil samples were air-dried, ground and mixed with 50 % (v/v) of sterile river sand. Per soil sample ten (1988) or six (1989) pots were filled with 200 ml of this mixture. In each pot one sugar beet seedling (cv. Regina) was planted. After a 6-week growing period in the greenhouse, sap was collected from the roots and analysed by ELISA.

Extension of inoculum sources measured in the second year. From the plots of the 1988 trial all plants were removed by hand in October. In November 1988, plots were cultivated to a depth of 20–25 cm with a spading machine used in horticultural crops (width 1.50 m), to avoid the sideward displacement of soil by ploughing. The seedbed was prepared using a power harrow and beet cv. Regina was sown again in 1989. Sowing distance was 18.5 cm with 50 cm between the rows. In September, all plants in 1 m^2 around the 1988 inoculum sources were analysed for the presence of BNYVV by ELISA. At a distance of 1 m from this square meter eight plants were taken and also analysed for BNYVV (distance to original source approximately 1.4 m).

II. Dispersal of inoculum by soil displacement.

Assessments in a field experiment

A field trial with different levels of inoculum, with or without irrigation, was laid out in 1988 (Tuitert and Hofmeester, 1992). Spread of BNYVV by means of infested soil was monitored yearly by analysing target plants at two distances from an infested area. As described for the complete trial, three of the four blocks were used for assessments. Behind the plots with the highest inoculum levels there were buffer zones (extra non-infested plots) in which mechanical operations ended to free the implements from infested soil (Figure 1 in Tuitert and Hofmeester, 1992). These plots were sown with beet similar to the other plots.

Plots were infested just before sowing in 1988. A tractor and a seed drill were the only machinery used, under dry conditions, after the inoculum was applied. In October 1988, target plants were sampled in the buffer zones at 2 and 8 m distance from the infested plot. The remaining plants were harvested by hand. In November 1988, plots were spaded as described before. Under dry conditions a power harrow was used to prepare the seedbed before sowing in 1989. In September 1989, plants were sampled again at 2 and 8 m from the infested plots. After spading in December 1989, seedbed preparation and sowing in April 1990, a third sampling was performed in October 1990.

Target plants were analysed for infection by BNYVV by means of ELISA using sap from the tips of the tap roots. The total number of plants examined per distance was 24 in all 3 years.

Results

1. Experiments with inoculum sources

Active dispersal in a greenhouse experiment
At 2 and 4 weeks after emergence of bait plants, none was infected by BNYVV at 5 and 10 cm from the inoculum sources. In the superficially infested control, all plants were infected after 4 weeks. Infection was absent in the non-infested control. The zoospore-emitting plants in the inoculum sources were all BNYVV-positive.

Spread of BNYVV from inoculum sources in the field
In none of the plots BNYVV was detected by bioassay in soil samples taken before the experiment started. In the single plot with a source of infested soil in between two rows, BNYVV-infected plants were not detected (Table 3). Distances between the inoculum source and the heart of the nine nearest plants in adjoining rows varied between 15 and 45 cm. In soil, BNYVV was detected at a distance of 20 cm from the source. BNYVV was not detected in soil around inoculum sources when there had been no target plants during the season (Table 3).

Detection of BNYVV-infected plants in the field. At the end of the growing season of 1988, two out of sixteen plants at 25 cm from an inoculum source were found to be infected by BNYVV, with low absorbance values in ELISA (Treatment 1, Table 2). In the other treatments, including the non-infested and the superficially infested plots, BNYVV-infected plants were not detected. Most plants growing in the inoculum sources showed rhizomania symptoms; all were highly positive in ELISA. In 1989, in none of the treatments BNYVV-infected plants were found. The only plants infected were those growing in the inoculum sources.

Detection of BNYVV in soil. Dispersal of BNYVV in soil had occurred at least over a distance of 25 cm in 1988 (Table 4), and perhaps over 50 cm. When the plant-free zone was 50 cm (Treatment 3), BNYVV was not detected at that distance. In 1989, dispersal had occurred over a distance of 25 cm (Table 4), but not when the inoculum source was without plants (Treatment 1). In Treatment 3 an additional assessment was made halfway between the plant-free zone and BNYVV was detected.

Extension of inoculum sources measured in the second year. The mean incidences of BNYVV-infected plants in 1989 around the inoculum sources of 1988 are presented in Table 5. Treatment effects on incidences were significant ($P < 0.001$) in the analysis of variance of angular transformed percentages. Treatment 1 was not different from the originally non-infested control (Treatment 4), but Treatments 2 and 3 were. In none of these treatments, the incidence of the completely infested plots (Treatment 5) was attained. At 1.4 m distance of the sources an occasional infected plant was detected (Table 5). Treatments 1 to 4 were not significantly different, only the superficially infested plots (Treatment 5) had a significantly higher incidence.

Table 4. Detection of BNYVV in soil at different distances from inoculum sources in the field. Experiments performed in 1988 and 1989. Four plots per treatment per year. One soil sample per sampling distance per plot. Bioassays with ten (1988) or six (1989) plants per soil sample.

Treatment[a]	Presence (+) or absence (–) of BNYVV-infected plants in bioassay[b]					
	1988			1989		
	25 cm	50 cm	plot area	25 cm	50 cm	plot area
1	+ (3/40)	+ (1/40)		– (0/24)	– (0/24)	
2	+ (3/40)	– (0/40)		+ (4/24)	– (0/24)	
3		– (0/40)		+ (1/24)	– (0/24)	
4			– (0/40)			– (0/24)
5			+ (16/40)			+ (4/24)

[a] Circular inoculum source for Treatment 1 was soil and for Treatments 2 and 3 soil with plants. Distances to target plants were 25, 25 and 50 cm, for Treatments 1, 2 and 3, respectively. Treatments 4 and 5 were non-infested or superficially infested with soil, respectively.
[b] Proportion of infected plants in bioassay given in parentheses.

II. Dispersal of inoculum by soil displacement

The effects of displacement of BNYVV-infested soil as shown by the incidences of infected plants are given in Table 6. In 1988, infection by BNYVV was not detected. In 1989, many plants were infected. Analysis of variance was performed on angular transformed percentages of infected plants, considering the split-plot design of the trial. The difference between the incidence of infected plants at 2 and at 8 m was significant ($P < 0.05$). In 1990, the plots were totally diseased. The apparent effect of irrigation on the expression of the displaced infested soil in 1989 was not significant ($P = 0.09$) at the confidence level applied.

Table 5. Incidences of BNYVV-infected plants in 1989 in 1 m^2 around the original inoculum sources of 1988 (diameter 20 cm, depth 12 cm) and at a distance of 1.4 m thereof.

Treatment	Type of inoculum source in 1988[a]	Distance of target plants in 1988	Proportion of BNYVV-infected plants in 1989[b]	
			Area 1 m^2	Distance 1.4 m
1	Soil without plants	25 cm	3/33 (9%) a	2/32 (6%) a
2	Soil with plants	25 cm	9/35 (26%) b	1/32 (3%) a
3	Soil with plants	50 cm	13/37 (35%) b	4/32 (13%) a
4	Non-infested	–	1/30 (3%) a	0/32 (0%) a
5	Superficially infested	–	29/38 (76%) c	20/32 (63%) b

[a] Treatments 1, 2 and 3 had circular inoculum sources, in Treatment 5 infested soil was spread evenly over the surface of the whole plots.
[b] In parentheses: arithmetic mean percentage of BNYVV-infected plants, determined by ELISA. Treatments were significantly different in ANOVA of angular transformed percentages ($P < 0.001$, for both area and distance assessments). Treatment means with the same letter are not significantly different ($P < 0.05$) according to Duncan's new multiple range test (Duncan, 1955).

Table 6. The incidence of BNYVV-infected plants at two distances from infested plots in the direction of the mechanical tillage operations. Assessments in three consecutive beet crops following artificial infestation of the plots in 1988[a].

Year	Number of BNYVV-infected plants[b]	
	2 m	8 m
1988	0 (0%)	0 (0%)
1989[c]	17 (71%)	10 (42%)
1990	23 (96%)	23 (96%)

[a] The trial was arranged in a split-plot design with drip irrigation levels (two) as main plots (Tuitert & Hofmeester, 1992).
[b] Number of plants tested per distance per year was 24. In parentheses: arithmetic mean percentage of BNYVV-infected plants.
[c] The difference between the two distances was significant ($P < 0.05$), the effect of irrigation was not significant ($P = 0.09$), and there was no interaction according to ANOVA of angular transformed percentages. In non-irrigated and irrigated plots the mean numbers of infected plants out of 24 (averaged over the two distances) were 10 and 17, respectively.

Discussion

Active dispersal of viruliferous P. betae. Spread of BNYVV in soil by active dispersal of vector propagules appears to be restricted by the limited distance covered by zoospores of *P. betae*. Relatively large distances, compared to the dimensions of a zoospore, were investigated. When the distance between infected 'source' roots and target roots was 5 cm, transmission of BNYVV to target roots was not detected. Under the prevailing conditions with fine sand, a fluctuating soil water potential and no watering from above, the distance of migration of viruliferous zoospores, resulting in infection, apparently was less than 5 cm. The presence of BNYVV in the roots of the infected source plants was verified, but the efficiency of the source plants as zoospore 'emitters' was not tested. In order to check the zoospore production and infection potential of the source plants during the experiment, a treatment could have been added with source plants surrounded by healthy target plants planted at different times. Application of a zoospore suspension to soil, instead of using infected plants as zoospore sources, would perhaps have resulted in migration of the zoospores over larger distances because of the absence of nearby source roots to re-infect. Soil water potential fluctuated between –3 and –6 cbar but fulfilled the requirements for infection of *P. betae* as determined in pot experiments (Gerik et al., 1990; De Heij, 1991). Literature on autonomous zoospore movement of other fungi shows that a few centimeters can be covered, 2–4 cm for *Phytophthora cryptogea* (Duniway, 1976), 4 cm for *Olpidium brassicae* (Westerlund et al., 1978), and that soil texture and soil moisture influence the distance.

At 5 and 10 cm distance from an inoculum source consisting of infested soil, BNYVV-infected plants were not detected. From this observation it may not be deduced that resting spores in soil were not induced to germinate by exudates of target roots at these distances, because zoospores, if released, could not overcome a distance of 5 cm. In general, the effect of root exudates on fungal propagules is limited to small distances from the root surface; from 1.5 and 3 mm for *Pythium ultimum* (Johnson and Arroyo, 1983) and *Phytophthora cinnamomi* (Zentmeyer, 1961), respectively, up to 10 mm for *Sclerotium cepivorum* (Coley-Smith, 1960) and 12–16 mm for *Gaeumannomyces graminis* (Gilligan and Simons, 1987).

Spread of BNYVV from inoculum sources in the field. For one soil type, two different years and in the absence of tillage and tread, it was shown that active dispersal, in combination with root growth, contributed only little to the horizontal dispersal of inoculum and spread of disease during the season. At 25 cm from an inoculum source, either infested soil or infested soil with plants, BNYVV was detected in only 2.5% of all plants sampled at that distance. At 50 cm, BNYVV-infected plants were not detected at all. In a single plot with a 20-cm-diameter source of infested soil between two rows at 50 cm distance, infected plants were not even detected in the adjoining rows (Table 3).

As it was surmised that roots could have become infected too late in the season to detect the virus in the tap root of the plant, soil samples were taken at the target distances after plants were removed, and assayed for viruliferous inoculum. The observations on root growth patterns by Brown and Biscoe (1985) supported the assumption that root contact of target roots with infested soil or the intertwining of infected and target roots can cause spread of the disease later in the season. Root density mid-way between rows (at 50 cm distance) was shown to be equal to root density close to the plant by mid-June (Brown and Biscoe, 1985). Nagata (1970) also observed an extension of roots till mid-way between the rows from July onwards. During the first two months after sowing, lateral roots extended only 5 cm horizontally (Nagata, 1970; Brown and Biscoe, 1985).

A bioassay on the soil samples revealed the presence of viruliferous *P. betae* at a distance of 25 cm from the inoculum sources (Table 4). When an area of 50 cm around the source had been kept free of plants, BNYVV was also detected at 25 cm distance (in 1989). This observation might suggest a role for root growth from the inoculum source in dispersal of inoculum, a suggestion in line with the failure to detect BNYVV at that distance around plant-free inoculum sources in fallow plots (Table 3). The role of root growth in the build-up and spread of inoculum was also evident from the disease incidence recordings in the second year after creating the inoculum sources. Disease incidence in 1 m^2 around the initial sources in which plants had been growing was significantly higher than in the absence of plants in the source (Table 5). Still, the incidences around sources were lower than those recorded in plots where inoculum had been applied to the whole surface. A trace of infestation, one out of 40 bioassay plants was BNYVV-positive with a low absorbance value in ELISA, was found at 50 cm distance when only infested soil was the inoculum source (Table 4). It is doubted whether this single observation should be ascribed to dispersal through the soil, because of the low incidence of disease in this treatment in the second year (Table 5). Besides, in 1989 BNYVV was not even detected at 25 cm in this treatment, although here the lower infestation level of the point source might have played a role. The latter may also explain why BNYVV was not found at 25 cm distance in the single fallow plot where plants grew in the inoculum source (Table 3). It should be added that environmental circumstances differed in both years. The warm summer of 1989, combined with a lower frequency and amount of precipitation compared to 1988, led to relatively dry soil moisture conditions, not favouring development of *P. betae*.

Epidemiologic implications. The results obtained indicate that secondary infection of a plant is not likely to occur from one row to another, current row spacing being 50 cm. The usual sowing distance within the row is 18.5 cm. A treatment with an infected plant within the row was not included and distances smaller than 20–30 cm were not investigated. And although viruliferous inoculum could be detected at 20–25 cm distance from a point source, detectable infection (BNYVV in tap root, occurrence of symptoms) of a plant by adjacent plants is not expected to play an important role in the increase of disease during the season. Therefore, it is suggested that the increase in disease incidence in the plant

population will depend on infection by inoculum (resting spores) present in the soil rather than on plant-to-plant spread of secondary zoospores.

Thus, although inoculum multiplies during the season by means of several secondary infection cycles, whereby the increase in number of infections on roots of individual plants is a polycyclic process, the hypothesis is put forward that epidemic development of rhizomania in the field will generally be a monocyclic process ('simple interest disease'; Van der Plank, 1963).

The situation may be different under very wet conditions (heavy showers, irrigation), depending on the soil type and the slope of the field (Shew, 1987), when transport of zoospores by runoff water over the surface or through soil pores (Wilkinson et al., 1981) will be more frequent. Besides having a direct moisture effect as just mentioned, irrigation may increase secondary spread of the disease because it favours root growth in the upper soil layers (Brown et al., 1987; Wild and Russell, 1988). Plant density will influence the frequency of plant-to-plant spread by changing the moment and intensity of root contact.

An increase in disease incidence in the field, resulting from conditions favouring the secondary infection cycle of *P. betae* and virus multiplication in the plant, should not be ascribed to spread, but primarily to recurrent infection of the same plant, an increasing virus content of the plant and/or an increased frequency of primary infections of the growing root mass exploring a larger volume of the infested soil.

Literature data on field experiments that confirm or negate these results and hypotheses stated are not available. The statement of Thresh (1986) on spread by soil-borne fungal vectors still applies: 'there is little information on the pattern and sequence of spread in crops, or on the relative amounts of spread over long and short distances'.

Dispersal of inoculum by soil displacement. In plots where circular inoculum sources were created in 1988, only few infected plants were detected at 1.4 m distance from the sources in 1989 (Table 5). Incidences of infected plants in these plots were not significantly different from those in the originally non-infested, and still disease-free, plots. Without tillage, viruliferous inoculum was detected at 25 cm from the sources in 1988. Tillage practices performed, spading, harrowing and drilling, were most likely responsible for displacement of infested soil resulting in diseased plants at further distances in 1989.

With whole-plot infestations, soil displacement can have more remarkable effects than with relatively small sources, as is apparent from the high incidences of infected plants at 2 and 8 m distance from infested plots in 1989 (Table 6). In 1988, BNYVV-infected plants were not detected at 2 and 8 m behind infested plots in the field trial with different inoculum levels. Nevertheless, some inoculum might have been present, presumably due to dispersal by wind, at the time of application of the infested soil to the plots, and possibly through displacement by tractor and seed drill and by animals. In 1989, infected plants were detected at both distances, showing that high inoculum levels were present. This infection will have been the result of multiplication in 1988 of the inoculum dispersed in 1988 as mentioned before, and of inoculum transported from the infested plots by tractor and spading machine in October 1988. Harrowing and sowing in spring 1989 were performed under dry conditions, limiting the displacement of soil. Plots were harvested by hand, and inside the buffer zone plots inoculum may have been dispersed by treading. An indication of the role of displacement of soil, most probably by the tillage in October, is given by the marked decrease in disease incidence with distance from the infested plots. Samples at larger distances were not included but, in view of the already strongly manifest level of infestation at 8 m, one can surmise that infested soil was distributed even further,

in spite of the limited tillage practices performed (e.g. no mechanical harvest).

Schäufele et al. (1985) recorded spread of inoculum of BNYVV in a particular field in the cultivation direction. Within 5 years, during which two sugar beet crops were grown and an intensive soil tillage was performed, BNYVV inoculum was detectable in soil samples throughout a 0.5-ha field, having overcome 60 m distance from the edges of the field to the centre. This observation will not have been the result of spread only. Multiplication of inoculum might also have been involved. It has been demonstrated in an artificially infested field that inoculum levels below the detection level of a bioassay and causing very low disease incidences in the field can increase to detectable and harmful levels after one or two beet crops (Tuitert and Hofmeester, 1992; Tuitert and Hofmeester, in preparation). In order to study the extent of spread of soil adhering to machinery, Hofmeester and Van Dullemen (1989) simulated a pathogen-infested area in a field crop by adding cress seeds to 1 m^2 just before the harvest of beets or potatoes. By observing the cress seedlings they assessed spread of 'infested' soil by harvesting machines up to 8 and 16 m distance and in small amounts even at 64 m distance from the original source.

The effect of irrigation on disease incidence in 1989 was too small to be significant, but should not be completely ignored (P=0.09). Irrigation may have influenced disease incidence in two ways. First, irrigation caused an increase in inoculum levels during 1988 (Tuitert and Hofmeester, 1992) and, therefore, soil transported from the irrigated plots had a higher inoculum level. Second, irrigation may have stimulated the development of disease during 1989.

After three beet crops, the area up to 8 m from the infested plots was heavily diseased, demonstrating once more the risks of spread of infested soil and the rapid multiplication of inoculum (Tuitert and Hofmeester, 1992).

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Chapter 8

Effects of sugar beet cultivars with different levels of resistance to beet necrotic yellow vein virus on the transmission of virus by *Polymyxa betae*

Effect of sugar beet cultivars with different levels of resistance to beet necrotic yellow vein virus on transmission of virus by Polymyxa betae

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Abstract

The effect of resistance of sugar beet cultivars to beet necrotic yellow vein virus (BNYVV) on virus content of resting spore clusters of the vector *Polymyxa betae* was studied in controlled environments and in naturally infested fields. The total number of resting spore clusters formed in roots of a partially resistant and a susceptible cultivar did not differ when assessed 6 and 12 weeks after inoculation with viruliferous resting spores. Transmission experiments showed that in partially resistant plants, having a low virus content in the roots, the population of resting spores formed was less viruliferous than that in susceptible plants with a high virus content. Consequently, growing a resistant cultivar can be expected to delay the build-up of virus inoculum in soil.

In a trial field sampled in 1991, the inoculum potential of BNYVV (most probable number of viruliferous *P. betae* propagules) in soil was lower after growing a partially resistant cultivar than after growing a susceptible one. On the other hand, in four sites sampled in 1990, inoculum potential in soil was hardly increased by growing sugar beet and was not significantly affected by the cultivar grown.

Additional keywords: *Beta vulgaris*, bioassay, BNYVV, inoculum potential, population dynamics, rhizomania, soil-borne virus, viruliferous population, virus vector

Introduction

The practical remedy against the devastating effects of rhizomania disease of sugar beet, caused by beet necrotic yellow vein virus (BNYVV), is the use of resistant cultivars. Breeding companies have developed several cultivars that perform well on infested fields, they aim for improving levels of resistance against the virus. Selection and evaluation of these cultivars is mostly done on the basis of their performance in the field (Richard-Molard, 1987). The so-called 'field resistance' or 'field tolerance' is often indicated by the yield of resistant or tolerant lines relative to a susceptible standard (Winner, 1988). In several of these lines a lower virus content than in susceptible ones was reported, indicating partial resistance to the disease (a.o. Giunchedi et al., 1987; Bürcky and Büttner, 1985 and 1988; Paul et al., 1992). Partial resistance to rhizomania in *Beta vulgaris* is based on resistance to the virus. Vector resistance does not seem to be involved, as in roots of the partially resistant cultivars high levels of infection by the

vector *Polymyxa betae* were observed (Asher and Barr, 1990). Complete resistance to the virus, or immunity (Cooper and Jones, 1983), has not been reported in *B. vulgaris*. Yield parameters were correlated with virus content of cultivars in greenhouse assays (Giunchedi et al., 1985; Bürcky and Büttner, 1991; Paul et al., in press).

Multiplication of BNYVV occurs in root cells and, as far as known, not inside the vector. *P. betae* acquires the virus during formation of zoosporangia and resting spores in the root cells. Viruses transmitted by plasmodiophorid vectors are thought to be carried internally, because the viruses survive drying and chemical treatment of resting spores and are still transmitted by zoospores in the presence of antiserum (Adams, 1991). In electron microscope studies, direct evidence of virus particles occurring inside zoospores was obtained for *P. betae* with BNYVV (Abe and Tamada, 1986) and for *P. graminis* with BaMMV (Jianping et al., 1991). Recently, Rysanek et al. (1992) showed the presence of BNYVV particles in both zoosporangial and cystogenous plasmodia, fungal structures developing into zoosporangia and resting spore clusters, respectively.

The reduced virus content in root cells of a resistant cultivar might be expected to reduce the number of particles acquired by the vector compared to those acquired in roots of a susceptible cultivar. Zoospores of *P. graminis* released from roots of a barley cultivar that was considered to be immune to barley yellow mosaic virus, transmitted the virus only rarely (Adams et al., 1987).

A reduced acquisition of virus by the vector from resistant cultivars could have important epidemiological implications. The build-up of viruliferous inoculum, which depends on the ratio of newly formed viruliferous resting spores and those that have germinated during the growing season, might be reduced by growing a resistant crop. The virus content of the vector population might decline.

We tested this hypothesis by assessment of effects of a partially resistant and a susceptible sugar beet cultivar on the viruliferous proportion of the population of resting spores. In this paper, we report on experiments in which we estimated the transmission of BNYVV by newly formed resting spores of either host. Besides, inoculum potentials of BNYVV (Tuitert, 1990) were estimated in soil samples taken in the field before and after growing of two cultivars with different susceptibility to the virus.

Materials and methods

Experiments 1 and 2. Effect of host plant resistance to BNYVV on virus content of newly formed resting spores

Partially resistant (R, cv. Rizor) and susceptible (S, cv. Regina) sugar beet plants were cultivated in infested soil. Resting spores were isolated from the infected roots of these source plants. Virus transmission was assessed by adding various numbers of resting spores to sterile coarse sand in which a susceptible test plant (cv. Regina) was grown. The numbers of BNYVV-infected test plants reflect the level of transmission of BNYVV by zoospores from the resting spores with different origin. The use of dilution series of resting spore suspensions enabled estimation of most probable numbers (MPN) of infective units, which were divided by known numbers of added resting spore clusters, yielding transmission (BNYVV) and recovery (*P. betae*) ratios. Experimental data are described below and given in Table 1.

Cultivation of plants on BNYVV-infested soil. Three-week-old sugar beet seedlings of cv. Rizor and cv. Regina were planted in a mixture of 5% (v/v) naturally infested sandy

Table 1. Data of transmission experiments with small tubes in the growth chamber (Expt 1 and Expt 2). In each experiment, two resting spore suspensions per cultivar were tested (two replicates). Both experiments were performed twice (A and B).

			Expt 1	Expt 2
Cultivation of source plants				
Number of plants per pot			3	1
Growing period greenhouse (weeks)			8	11
Absorption values (x1000) in ELISA on BNYVV [a]				
Tap root / laterals				
Source plants:	R-1 [b]		5/ 33	1/ 10
	R-2		21/ 88	3/ 13
	S-1 [b]		724/457	329/653
	S-2		703/309	1003/791
	CR (control R)		1/ 11	4/ 5
	CS (control S)		10/ 13	5/ 13
Resting spore inoculum [c]				
Clustersize (μm^2)				
R source:	mean ± s.d.		562 ± 307	448 ± 209
	median		489	419
	range		161-2460	109-1082
S source:	mean ± s.d.		600 ± 345	494 ± 233
	median		541	432
	range		109-2163	135-1217
Densities tested [d]		A:	10,100,1000	0.1,1,10,100
(numbers ml^{-1} sand)		B:	1,10,100,1000	1,10,100,1000
Assay of virus transmission				
Tube size (diameter x length, cm)			2 x 10	2.4 x 8.5
Amount of sand per tube (ml)			25	28
Temperature in climate room		- air	22-23	22-23
(°C)		- soil	24-25	24-25
Light (hours/intensity (lux))			16/±18,000	16/±18,000

[a] ELISA-absorption values of the two infected source plants per cultivar from which resting spores were extracted for assessment of virus transmission. CR and CS are the non-infected control plants of R and S, their background absorption values are below the approximate positive-negative threshold of 40-50.

[b] R = cv Rizor, S = cv Regina.

[c] Cluster size is approximated by the product of length and width of individual resting spore clusters. The only differences in size distribution were between the pooled data of Expt 1 and those of Expt 2 ($P<0.001$ for location; $P<0.05$ for dispersion), see text.

[d] The numbers of resting spores applied to test plants in order to assess virus transmission.

clay soil with coarse sand and placed in a greenhouse. The MPN of infective units, per 100 g of dry infested soil was estimated as 156 for BNYVV (viruliferous *P. betae*) and 1570 for *P. betae*, determined as described before (Tuitert, 1990).

Preparation of suspensions of resting spore clusters. Roots of the infected R and S plants from the greenhouse were washed free of soil in running tap water, all roots were collected; supernatant with little rootlets was decanted over two sieves (1 mm and 0.075 mm mesh). Lateral roots were separated from the tap roots. Sap samples, collected from lateral roots and from tap roots, were analyzed by ELISA for presence of BNYVV (Clark & Adams, 1977; Tuitert, 1990). Roots of all S plants had a high virus content, especially in their laterals. R plants had lower and variable virus contents, for some plants ELISA values were in the range of those of the non-infected control plants, especially for the tap roots. For isolation of resting spores, plants were non-randomly selected. Two S plants (S-1, S-2) were taken which had a high ELISA value also for their lateral roots, the two R plants (R-1, R-2) were selected on the basis of their low ELISA values for both lateral and tap roots (Table 1). Lateral roots were cut into pieces with a scalpel, then macerated by pestle and mortar and suspended. The suspension was sucked through Monodur gauze (50 µm mesh) to remove large root debris, the filtrate was centrifuged and the precipitated resting spores suspended in small amounts of water (Tuitert, 1993). Densities of resting spore clusters in the suspensions were adjusted in order to enable addition of the desired numbers of spores in equal amounts of water to tubes filled with sand (Table 1). As in Expt 1 three source plants were grown in one pot, ELISA was performed on the pooled lateral roots of the three plants, and resting spore suspensions were made of these triplets. The resting spore suspensions of Expt 2 originated from single plants.

Resting spore clusters differ in the number of individual resting spores. As the size of the clusters might influence transmission - a large cluster can release more viruliferous zoospores than a small one - size distributions of clusters in the suspensions were determined. Lengths and widths of spore clusters were measured and their products (projected cluster areas) were used to characterize cluster size. From each of the four resting spore suspensions in Expt 1, 100 clusters were selected at random and measured, from each of the suspensions of Expt 2, 50 clusters were taken (Table 1). The form and location of the frequency distributions of resting spore sizes were compared by means of the nonparametric Kolmogorov-Smirnoff two sample test and the Mann-Whitney U test, respectively (Siegel, 1956; Payne et al., 1991).

Assay of virus transmission. Pasteurized coarse sand (80°C, c. 18 hours) was dried and sieved (2 mm). The density of the sand was approximately 1.6 g ml^{-1}, the pH was between 6.5 and 7.0. Perspex tubes, one side open, were carefully filled with sand and placed in foam holders in the growth chamber (Table 1). Resting spores were added in 1 ml suspension to the tubes, yielding the densities as given in Table 1. For the control series, diluted root sap from non-infected plants was used. The tubes were left to air-dry for two weeks, in order to kill zoospores and zoosporangia (Campbell & Lin, 1976). After moistening the sand by addition of 2 ml of 50% Steiner nutrient solution, two-week-old susceptible sugar beet seedlings (cv. Regina) were planted in the tubes. The small planting sticks used were carefully cleaned above the sand by means of a fixed volume of demineralized water, to avoid removal of adhering resting spores from the tube. Every other day, water and 50% Steiner nutrient solution were added alternately to the top of each tube by means of a dispenser. After four weeks, the soil in the tubes was soaked in water and roots of the seedlings were gently washed out. The presence of *P. betae* and BNYVV in the rootlets was determined according to Tuitert (1990). The numbers of infected plants were used to estimate the MPN of infective units of both vector and virus in the infested sand. Differences between MPNs were tested according

to Cochran (1950).

Expt 3. Effect of host plant resistance to BNYVV on numbers of resting spores of P. betae formed

In a third experiment, root weight and development of *P. betae* in roots of R and S plants at two inoculum levels of BNYVV, created by addition of resting spores, were investigated.

Preparation of inoculum. Resting spores were isolated from the rootlets of 20 sugar beet plants cv. Regina grown for 5 weeks in a 10% mixture (v/v) of soil naturally infested with BNYVV (as described before) and sterile coarse sand. All plants were BNYVV-positive in ELISA, absorption values (x1000) of individual tap roots ranged from 96 to 1744; for a pooled sample of lateral roots it was 1554. Lateral roots were dried during 2 days at 37 °C. They were then treated as described before to prepare resting spore suspensions with densities adjusted to enable addition of two different numbers of spores in equal amounts of water to the assay plants. For the control series, diluted root sap from non-infected plants was used.

Design of the experiment. Two-week-old plants of cv. Rizor (R) and cv. Regina (S) were planted individually in pots filled with 200 ml sterilized coarse sand (pH 6.5). By means of a hypodermic syringe resting spores were injected into the moistened sand in a circle around each seedling. The inoculum levels created were: 0, $2x10^3$ and $1x10^6$ resting spore clusters per pot (20 pots per inoculum level). Pots were placed randomly in a growth chamber at 22 °C during daytime (16 hours) and 15 °C at night.

Assessment of infection. After 6 and 12 weeks, roots of ten plants per inoculum level were carefully washed free of the sand and all roots were collected by means of sieving and decanting. Lateral rootlets were separated from the tap root, cut to pieces of c. 0.5 cm and suspended in demiwater. Root pieces were collected by sieving through Monodur gauze (50 µm mesh) on a Buchner funnel. The gauze with root pieces was blotted dry and fresh weights of laterals and tap roots were recorded. A sample of 500 mg of lateral roots from each plant was taken for determination of the number of resting spores present. After addition of 1 ml 1.5 M KOH solution (after Goffart et al., 1987), the root sample was macerated with pestle and mortar during 2 min and then kept for 2 h at room temperature in a potter tube. The suspension was homogenized in the glass tube for 1 min and the volume was then adjusted to 10 or 15 ml. The density of resting spores was determined. The remainder of the lateral roots was used for ELISA. Besides, sap was extracted from the tap root for ELISA.

Expt 4. Field trials

In the field, the effect of host plant resistance to BNYVV on the inoculum level in soil was studied by assessing populations of virus and vector before ('initial', Pi) and after ('final', Pf) growing a partially resistant and a susceptible cultivar. We participated in current cultivar trials on rhizomania-infested fields conducted in 1990 and 1991 at different locations in the Netherlands (Table 2). The numbers of cultivars or lines ranged from 16 to 20, the trials were laid out in four blocks. From the available cultivars, 'Rima' and 'Univers' were chosen to represent a partially resistant and

susceptible cultivar, respectively. Soil samples were taken between the rows, soon after emergence of the crop and after harvest in the beginning of the following year (Table 2). Each plot was sampled with a frequency of 1 core per square meter. The cores, 1.3 cm diameter to a depth of 25 cm, of one plot were mixed to form one compound sample. Treatment of soil samples and assessment of the inoculum potential of *P. betae* and BNYVV followed Tuitert (1990).

Table 2. Data on field trials where inoculum potentials of BNYVV and *P. betae* were determined in the soil before and after growing a partially resistant ('Rima') and a susceptible cultivar ('Univers').

Code	Year	Location	Plot size (m^2)	Sampling date [a]		Soil parameters		
				Pi [b]	Pf [b]	pH-KCl	O.m. [c]	$CaCO_3$ [c]
LZ90	1990	Lage Zwaluwe	6 x 7	01-05-90	29-01-91	7.3	3.5	6.3
NOPV90	1990	Nagele 'V'	6 x 7	02-05-90	07-03-91	7.3	4.5	7.6
ARN90	1990	Arnemuiden	6 x 7	01-05-90	30-01-91	7.3	2.2	9.5
NOPB90	1990	Nagele 'B'	6 x 7	02-05-90	22-03-91	7.2	4.5	7.9
THO91	1991	Tholen	3 x16	03-05-91	09-03-92	7.4	3.1	3.4

[a] Four plots per cultivar were sampled at every location.
[b] Pi='initial' population, Pf='final' population.
[c] Organic matter (O.m.) and $CaCO_3$ given as a percentage of dry soil.

MPNs of infective units of BNYVV or *P. betae* per 100 g of soil were $\log_{10}$-transformed ($\log_{10}$ MPN + 1) before analysis. Multiplication ratios were also transformed ($\log_{10}$). Percentages viruliferous *P. betae* were estimated by division of MPNs of BNYVV by those for *P. betae*. Data of the sampling after harvest (Pf) were tested by analysis of covariance, using the initial MPNs in spring (Pi) as covariates, because multiplication of BNYVV was shown to depend on inoculum level (Tuitert and Hofmeester, 1992). Calculations were performed for the separate locations by means of Genstat 5 (Payne et al., 1988).

Results

Transmission of BNYVV by newly formed resting spores (Expt 1 and 2)

In both Expt 1 and Expt 2, all susceptible source plants were positive in ELISA, indicating that sufficient inoculum was provided in the 5%-infested soil mixture to infect all plants.

Two checks were made to assess the effect of factors, other than host resistance, that might influence the transmission ratio of BNYVV by the resting spores; i.e. size of the resting spore clusters and their infectivity, measured by recovery. The resting spore suspensions originating from the R and S cultivars did not differ in size distribution and mean or median size of resting spore clusters within each experiment (Table 1, $P<0.05$). Mean cluster size was larger in Expt 1 than in Expt 2 ($P<0.001$). The bioassay yielded a

similar recovery of the introduced resting spore clusters, whether they originated from R or from S plants (Table 3). One to two percent of the added resting spores clusters were 'recovered'. Thus, one infective unit of *P. betae* corresponded with 50-100 clusters in the sand.

Table 3. Effect of host plant resistance to BNYVV on the percentage of resting spore clusters of *Polymyxa betae* that transmitted BNYVV and on the recovery of *P. betae*. Most probable numbers (MPNs) of BNYVV and *P. betae* per ml soil were estimated for the sand samples infested with 1000 spore clusters per ml from a partially resistant (R) or a susceptible (S) cultivar[a].

Expt	Cv.	BNYVV		Transmission ratio S/R[c]	*P. betae*		Viruliferous clusters (%)[e]
		MPN ml^{-1}	Transm. (%)[b]		MPN ml^{-1}	Recovery (%)[d]	
1A	R	0.5	0.05	10	7.6	0.8	6.7
	S	4.8	0.5		9.2	0.9	50
1B	R	0.6	0.06	15	24.6	2.5	2.5
	S	9.1	0.9		24.6	2.5	37
2A	R	0.02	0.002	120	6.0	0.6	0.003
	S	2.3	0.2		8.3	0.8	32
2B	R	0.003	0.0003	990	8.0	0.8	0.04
	S	3.0	0.3		8.9	0.9	34

[a] MPNs were based on assessments of 20 plants per spore density. Spore densities tested are given in Table 1. Within every experiment, MPNs of *P. betae* for R and S were not significantly different ($P<0.05$, Cochran, 1950), but MPNs of BNYVV were significantly different ($P<0.001$) for the R and S origin.
[b] The percentage of clusters which transmitted BNYVV ((MPN BNYVV / 1000)x100).
[c] MPN BNYVV of S divided by MPN BNYVV of R.
[d] The percentage of clusters which was infective, or recovered ((MPN *P. betae* / 1000)x100).
[e] MPN BNYVV divided by MPN *P. betae* (x100).

There were no significant differences in MPNs between the two suspensions of each cultivar (R-1 and R-2, S-1 and S-2). Therefore the mean values for the duplicates are presented (Table 3). MPNs of BNYVV differed between R and S ($P<0.001$), for every experiment; resting spores from R plants transmitted less virus than those from S plants. The transmission ratio between S and R was estimated as 10 and 15 in Expt 1 up to 120 and 990 in Expt 2.

Estimates for the proportion of viruliferous clusters ranged from 32 to 50% for those originating from S plants, to less than 1% up to 6% for those from R plants (Table 3).

Density and total number of resting spores in rootlets of resistant or susceptible plants at two inoculum levels of viruliferous P. betae (Expt 3)

After 6 weeks, there were no significant effects ($P=0.05$) of cultivar and inoculum

level on the density of resting spore clusters per mg of root and on the total number of resting spore clusters formed. The resting spore density was $4.1x10^3$ (mean $\log_{10}$ value = 3.61 ± 0.10) per mg of root; the total number of resting spores formed per plant (density multiplied by root weight) was $4.7x10^6$ (mean $\log_{10} = 6.68 \pm 0.10$). A multiplication ratio of *P. betae* was estimated by dividing the total number of resting spore clusters formed by the number added to each plant. Multiplication was the same for both origins (R and S), but was highest at the lowest inoculum level: ratios of 2023 and 6 at inoculum levels of $2x10^3$ and $1x10^6$ spores per seedling, respectively. Irrespective of the cultivar tested, after 6 weeks fresh weights of lateral roots and tap roots were significantly reduced and ELISA-values raised by the viruliferous inoculum added.

After 12 weeks, the number of spores per mg of root tended to decrease at the highest inoculum level, geometric mean density was $5.2x10^3$ per mg compared to $7.6x10^3$ per mg at the lowest inoculum level, but the difference was not significant (Table 4). There was still no difference between cultivars in density or total number of spores per plant. The overall mean density was $6.2x10^3$ per mg root ($\log_{10} = 3.79 \pm 0.06$). The total number of spores formed per plant was $13.3x10^6$ (7.12 ± 0.06). The multiplication ratio was highest at the lowest inoculum level (Table 4).

Table 4. Density of resting spore clusters, multiplication ratio of *Polymyxa betae* in lateral roots, root fresh weights and ELISA-absorption values of BNYVV in tap and lateral roots of sugar beet seedlings differing in resistance to BNYVV. Seedlings were grown for 12 weeks in sand infested with viruliferous resting spores (Expt 3).

Inoculum level or cultivar [a]	*P. betae* spore clusters[b]		Root fresh weights $\log_{10}$ (g)[b]		ELISA-values, $\log_{10}$ (absx1000)[b]		
					Tap root		Laterals
	Density $\log_{10}$ n mg^{-1}	Multiplication ratio, $\log_{10}$	Tap root	Laterals	R	S	
0	-	-	0.57 (3.69)	0.26 (1.80)	1.00 (9)	0.58 (4)	1.36 (22)
$2x10^3$	3.88 (7600)	3.90 (7850)	0.48 (3.01)	0.40 (2.52)	1.60 (39)	2.13 (132)	2.30 (196)
$1x10^6$	3.72 (5200)	1.05 (11)	0.21 (1.61)	0.37 (2.32)	2.00 (100)	2.94 (862)	2.93 (844)
P(inoc)[c]	0.12	<0.001	<0.001	<0.001	<0.01	<0.01[d]	<0.001
LSD(0.95)	0.21	0.23	0.15	0.07	0.36	0.51[d]	0.36
R	3.80 (6250)	2.44 (280)	0.46 (2.87)	0.29 (1.95)	1.53 (33)		2.09 (124)
S	3.80 (6250)	2.50 (320)	0.38 (2.39)	0.39 (2.44)		1.88 (75)	2.30 (197)
P(cv)[c]	1.00	0.59	0.19	<0.01	<0.05		0.18

[a] The number of spore clusters injected around each seedling. The partially resistant cultivar Rizor (R) and the susceptible cultivar Regina (S) were used.

[b] Back-transformed means are given in parentheses.

[c] *P*(inoc) is the F-probability of the effect of inoculum level on the parameters in ANOVA, the LSD for comparison between the inoculum levels. Interaction was not significant, except for tap root ELISA ([d]). *P*(cv) refers to the cultivar main effect.

[d] The *P*-value of the interaction of inoculum level and cultivar, the LSD for comparison of inoculum levels between the two cultivars.

When plants had grown for 12 weeks in infested soil, lateral root weight was higher than in non-infested conditions ($P<0.01$). Apparently, cv. Rizor formed less lateral rootlets than cv. Regina during the 12-week growing period ($P<0.01$), independent of the three inoculum levels used (interaction between inoculum level and cultivar not significant at $P=0.05$). The difference in 12-week tap root weight between cultivars was not large enough to be significant.

After 12 weeks, virus content in tap roots, as indicated by the ELISA-absorption values, was higher in the susceptible cultivar, particularly at the highest inoculum level (Table 4).

Inoculum potentials of BNYVV and P. betae in field trials (Expt 4)

In five locations geometric mean inoculum potentials of BNYVV (viruliferous *P. betae*) ranged from 2 to 21 infective units per 100 g soil at the beginning of the growing season (Table 5). In one of the five locations, NOPV90, the pattern of infestation before the trial was patchy (which was reflected in the distribution of diseased plants later on), with inoculum levels of BNYVV ranging from below the detection level (<0.6) to an outlier of 87 (per 100 g soil). Estimates of the percentages of fungal propagules carrying virus ranged from 0.5% to 6% for the different locations (Table 5).

The Pi and Pf (adjusted for the Pi) of BNYVV and *P. betae* are presented in Figures 1 and 2. The statistical significances (*P*-values) of cultivar effects on Pf of BNYVV and *P. betae* at the five locations are given in Table 5. The Pf of BNYVV was significantly ($P=0.01$) lower after 'Rima' than after 'Univers' for THO91, and showed a trend to be lower ($P=0.054$) for LZ90.

Table 5. Significance (*P*-value) in statistical analysis of the effect of cultivar grown on inoculum potential of BNYVV and *Polymyxa betae* in soil and on the percentage of the population *P. betae* carrying virus. Geometric means of inoculum potentials of BNYVV in soil before (Pi) and after (Pf) growing of the partially resistant cultivar Rima (R) and the susceptible cultivar Univers (S) and arithmetic means of viruliferous percentages of *P. betae*.

Location	BNYVV*				Viruliferous *P. betae* (%)				Total *P. betae*
	Pi	Pf		*P*-value	Pi	Pf		*P*-value	*P*-value
		R	S			R	S		
LZ90	2.0	3.1	6.4	0.05	2.2	0.1	2.7	0.19	0.50
NOPV90	4.1	15	16	0.93	0.8	1.0	1.0	0.95	0.24
NOPB90	15	29	33	0.89	0.5	1.3	1.2	0.91	0.10
ARN90	21	54	36	0.53	3.2	2.2	4.1	0.17	<0.01
THO91	7.3	37	91	0.01	6.2	0.2	2.4	0.17	0.81

* Back-transformed mean $\log_{10}$-transformed MPNs per 100 g dry soil are presented, the Pf values were adjusted for the covariates (Pi).

Populations of total and viruliferous *P. betae* hardly increased at the locations investigated in 1990, only in THO91 did populations show a considerable increase

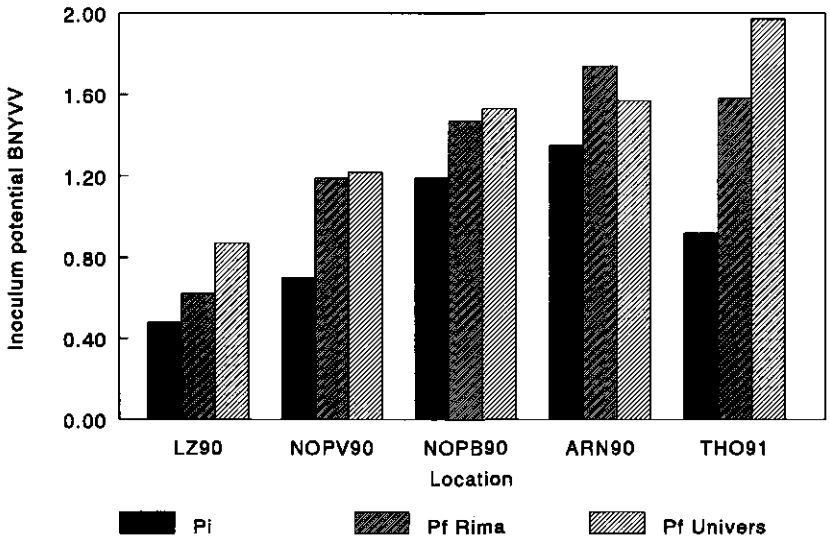

Fig. 1. The inoculum potential (log_{10} MPN +1) of BNYVV in soil, before (Pi) and after (Pf) growing a partially resistant (Rima) and a susceptible sugar beet cultivar (Univers) at five locations. Locations and significances of cultivar effects are described in Tables 2 and 5, respectively.

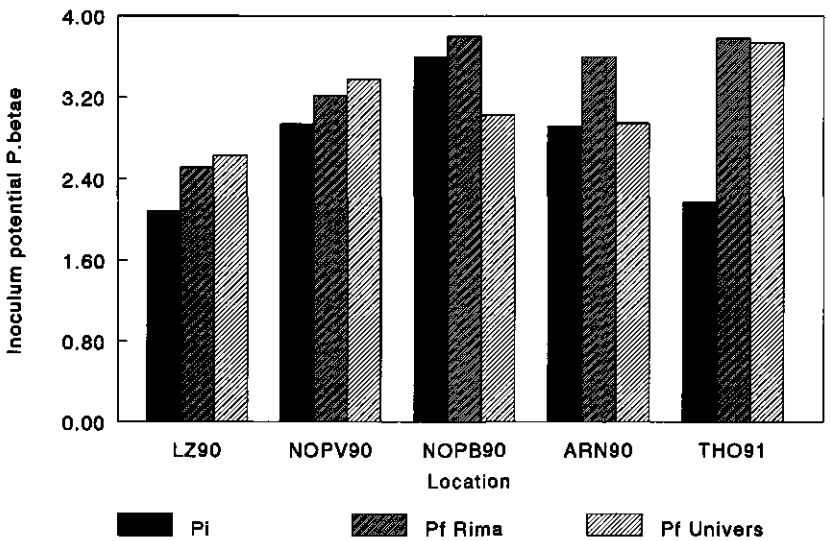

Fig. 2. The inoculum potential (log_{10} MPN +1) of *Polymyxa betae* in soil, before (Pi) and after (Pf) growing two sugar beet cultivars at five BNYVV-infested locations. Cv Rima is partially resistant to BNYVV, cv Univers is susceptible. Locations and significances of cultivar effects are described in Tables 2 and 5, respectively.

(Table 6). For THO91 and LZ90, the multiplication ratios of BNYVV with 'Rima' were lower than with 'Univers' (Table 6). Estimates of the viruliferous percentages of the population *P. betae* after 'Rima' were not significantly different from those after 'Univers' in THO91 (Table 5). For the location with the highest Pi of BNYVV, ARN90, the Pf of *P. betae* and the multiplication ratio of *P. betae* were lower after growing 'Univers' than after 'Rima'.

Table 6. Multiplication of BNYVV (viruliferous *Polymyxa betae*) and *P. betae* in soil under the partially resistant cultivar Rima (R) and the susceptible cultivar Univers (S).

Location	BNYVV			*P. betae*		
	Multiplication ratio[a]		*P*-value[b]	Multiplication ratio[a]		*P*-value[b]
	R	S		R	S	
LZ90	1.6	3.7	<0.01	2.7	3.6	0.50
NOPV90	4.9	3.9	0.82	1.9	2.7	0.24
NOPB90	2.0	2.3	0.74	1.6	0.3	0.10
ARN90	2.6	1.7	0.52	4.7	1.1	<0.01
THO91	5.1	13	0.01	41	38	0.81

[a] Back-transformed from mean $\log_{10}$ (multiplication ratio).
[b] *P*-values refer to the effect of cultivar on multiplication in analysis of covariance of the $\log_{10}$-transformed ratios.

Discussion

Recovery of resting spores. The MPNs calculated for *P. betae* were 1-2% of the numbers of spore clusters added, thus one infective unit of *P. betae* corresponded with 50-100 resting spore clusters in the sand. As individual resting spores have a diameter of 4.2-5 µm (Keskin, 1964) and the majority of resting spore clusters is rather 'flat', usually consisting of a single layer of spores (Barr, 1979; Ciafardini and Marotta, 1988), the average cluster could be estimated to consist of approximately 30 to 37 spores in Expt 2 and Expt 1, respectively. Apparently, cluster size did not affect 'recovery' of *P. betae* in these experiments, as no consistently (only Expt 1B) or significantly higher recovery was obtained with the suspensions of Expt 1 compared to those of Expt 2. The recovery percentage of *P. betae* in sand was the same as the one found in a previous bioassay procedure by Tuitert and Bollen (1993). In that paper, agreement with data from other authors was discussed in detail. As the recovery of *P. betae* did not differ significantly between the different origins, the transmission ratio of spores from the susceptible and partially resistant origin was not influenced by the infectivity of the spores.

Virus content of newly formed resting spores. It was shown that in roots of 8-11-week-old partially resistant sugar beet plants with a low content of BNYVV, less viruliferous resting spore clusters of *P. betae* were formed than in susceptible plants with a high virus content. The difference in transmission of BNYVV by resting spores of R and S plants was larger in Expt 2 than in Expt 1 (Table 3). This difference in the two

experiments corresponded with that in virus content in the lateral rootlets of the R source plants, which in Expt 2 (absorption values in ELISA comparable to those of the non-infected control plants) was lower than in Expt 1 (Table 1).

The level of resistance of cv Rizor to BNYVV was not sufficiently high to prevent the formation of viruliferous resting spores of *P. betae*. For that, immunity will be required (Adams et al., 1987). Canova (1966) reported that viruliferous isolates of *P. graminis* lost their ability to transmit soil-borne wheat mosaic virus after growth on a non-host (clover). The mechanism of acquisition of BNYVV by fungal structures inside virus-infected root cells has not been elucidated. It was shown that proteins encoded by RNA 2 and RNA 4 of BNYVV are associated with efficient transmission of BNYVV by *P. betae* (Tamada and Abe, 1989; Tamada and Kusume, 1991), and thus may be involved in acquisition.

Viruliferous fraction of the resting spore population in roots. Thirty to fifty percent of the infective propagules of *P. betae* was estimated to be viruliferous when originating from a susceptible plant, and less than 1% to 6% when originating from a partially resistant plant. For the resting spore clusters from susceptible BNYVV-infected plants used in infection experiments by Fujisawa and Sugimoto (1977), approximately 86% appeared to be viruliferous, as estimated by calculation of MPNs for *P. betae* and BNYVV, using the numbers of infected plants presented. Rysanek et al. (1992) estimated that in some cases at least 50% of plasmodia in roots contained BNYVV. In situ localization of BNYVV particles in mature resting spores has not yet succeeded (Rysanek et al., 1992). As a consequence, there is no information on the distribution of virus particles inside resting spore clusters. Probably not all individual resting spores in and zoospores released from a viruliferous cluster contain the virus. For *P. graminis*, only 1-2% of zoospores released from zoosporangia in roots of a susceptible plant were found to be viruliferous (BaMMV), as determined by electron microscope observations (Jianping et al., 1991). The failure to detect BNYVV in 220 mature zoospores of *P. betae* by Giunchedi and Langenberg (1982) might have been due to a low proportion of viruliferous ones.

The transmission percentage of BNYVV by the added clusters and the recovery of the clusters were estimated by taking the ratio of the MPNs, calculated on the basis of the number of infected plants at all dilutions, and the number of spore clusters added. With prior knowledge of the dilution endpoint of virus and vector, one dilution for either virus or vector could be used to estimate transmission percentages according to the method described for group testing of infected plant samples (Gibbs and Gower, 1960), insect vectors (Swallow, 1985), or seed transmission of virus (Maury et al., 1985). Percentages thus calculated were in good agreement with those presented in Table 3.

Density and total number of resting spore clusters in BNYVV-infected roots. The density of resting spore clusters in the roots and the total numbers formed per plant were not affected by resistance of the plant to BNYVV in a 6- and 12-week growing period. Consequently, a lower virus content of resting spores in R plants than of those in S plants would result in a lower population of viruliferous spores formed per R plant. In the field, a 100-fold reduced inoculum level could give a significantly higher yield (Tuitert and Hofmeester, 1994), depending on the level of infestation and environmental conditions.

After 12 weeks, lateral root weight was slightly increased in all infected plants. Early

and severe infection of beets by BNYVV can lead to the typically bearding symptom, caused by root necrosis and root proliferation. A susceptible cultivar develops a higher incidence of bearded beets than a partially resistant one. It is not known what the effects of these symptoms will be on multiplication of *P. betae* in older S plants, in comparison with multiplication in the more normally developed roots of older R plants. An increased multiplication of *P. betae* in the relatively healthy root system of R plants could compensate for the effect of reduction in total numbers of viruliferous spores.

The multiplication ratio of *P. betae* was highest for the lowest inoculum level (a 500-fold lower inoculum level had a 300-fold higher multiplication ratio). This negative correlation between inoculum level and multiplication ratio was also found for the viruliferous population of *P. betae* in soil samples from a field experiment (Tuitert and Hofmeester, 1992). The spore suspensions were injected around the plant and therefore not dispersed through the whole soil volume. Especially at the highest inoculum level, clumping of resting spore clusters may have reduced multiplication of individual clusters. Even so, considering recovery percentages found in other experiments (Table 3), the actual multiplication of individual resting spore clusters at both inoculum densities was probably 100-fold higher than theoretically calculated, considering that only 1% of the added clusters germinated.

Field levels of inoculum. In all trial fields of 1990, the increase in inoculum potential of BNYVV by growing sugar beet was small, multiplication was only two- to fivefold, irrespective of the initial inoculum level present. In this year, symptoms in rhizomania-infected crops were moderate. In 1991, only one field was examined and multiplication was 5- to 13-fold, with the smallest increase for the partially resistant cultivar Rima. Geometric mean inoculum potentials of BNYVV per location ranged from 2 to 21 infective units per 100 g of soil. Multiplication ratios at these mean inoculum levels were found to be 71 and 17, respectively, during one year (1989) on one location (Tuitert and Hofmeester, 1992). A larger number of 'location-years' should be studied to enable analysis of the relative importance of factors that determine multiplication.

The low multiplication ratios in 1990 were disadvantageous for a study on differences in multiplication between cultivars. Beside a low multiplication ratio, other aspects might have affected the results of soil sampling and assessment. The five trial fields described were situated on farmers' fields where no special precautions were taken to prevent dragging of soil by machinery or treading at harvest. However, the Pf-sampling was performed on smaller nett fields than the original ones in order to avoid border effects. Being ignorant of the spatial pattern of inoculum in the fields, we sampled with a high frequency. Immune cultivars are not available, and in the partially resistant cultivar a fraction of plants still has a high virus content (Paul et al., 1992), thus contributing to multiplication of viruliferous *P. betae* like infected susceptible plants. The contribution of newly formed resting spores to the resident population has to be large to be measured by the bioassay. A measurable decline in BNYVV levels in the soil will depend on the proportion of the viruliferous resting spores of the vector that germinate during the season. Perhaps, larger differences in Pf after R and S cultivars could be measured when the level of resistance in R plants is increased and when they are grown for more than one year at the same site. However, recent data from Adams et al. (1993) showed that growing a resistant barley cultivar or a non-host crop (wheat) for 3 years did not significantly reduce soil populations of barley mild mosaic virus, although a trend was apparent.

At all locations, the inoculum potential of *P. betae* in soil was high relative to that of

BNYVV, the viruliferous percentages of the population were estimated at 0.5-6%. A significant increase of *P. betae* (40-fold) occurred at only one out of five fields. At the location with the highest infestation with BNYVV, multiplication of *P. betae* was low on the susceptible cultivar. An explanation for this phenomenon might be that BNYVV-infection of the susceptible cultivar caused a net reduction of available infection sites, despite the bearding symptom of the roots, whereby *P. betae* had less opportunities for multiplication. Another explanation can be derived from the observations of Schlösser (1990), who found that a high concentration of BNYVV in newly formed roots on a diseased tap root inhibited infection of these roots by *P. betae*.

Conclusions. In conclusion the results can be summarized as follows. The finding that in roots of a partially resistant sugar beet plant with low virus content less viruliferous resting spores were formed than in roots of a virus-infected susceptible plant confirms the hypothesis that the fungal vector acquires less virus from virus-resistant plants than from susceptible ones. The prediction deduced from the stated hypothesis was a decline in virus content of the vector population, due to a reduced build-up of viruliferous inoculum in soil by resistant plants. A measurable difference in soil inoculum of BNYVV in the field after growing a partially resistant compared to a susceptible cultivar was found in the trial of 1991 and, to a lesser extent, in one out of four trials of 1990. A low multiplication ratio irrespective of the cultivar (probably partly due to a low proportion of soil-borne spores that germinate), the variability in the population assessments in samples from one field (partly due to the spatial pattern of inoculum in the field) and the absence of highly resistant or immune cultivars are factors that either hamper the study of differences in multiplication in the field situation or are responsible for the absence of measurable effects.

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Chapter 9

General discussion

General discussion

Quantifying inoculum of BNYVV and P. betae in soil

A method for quantitative detection of the pathogen population is indispensable to address questions on the epidemiology of a disease, such as the amount of inoculum necessary to cause disease and the changes in the pathogen population over time.

In the tripartite plant pathosystem that is the subject of this thesis, a virus (BNYVV), a vector (*Polymyxa betae*) and a host plant (*Beta vulgaris*) are involved. Detection of the vector in soil is hampered by the fact that it is an obligate parasite, which cannot be cultured in vitro without a host plant. A direct method of enumeration of its resting spores in soil has not yet been published, besides, such a method would have the disadvantage that it does not distinguish between dead and viable propagules. Direct detection procedures for the virus, present inside the resting spores of the vector, have neither been developed for detection in soil samples. Application of PCR (polymerase chain reaction) might enable detection of small amounts of virus RNA in soil after releasing the virus from the resting spores. However, both PCR and amplified ELISA are inhibited by soil components (Adams, 1993). Hence, for the time being, we rely on the use of a bioassay for detection of virus and vector in soil. Detection of BNYVV in (bait) plant roots can adequately be performed with ELISA. The vector is detected by microscopical examination. Development of molecular methods for detection of *P. betae* might make the assessment of fungal structures in the roots less time-consuming in future (Ward et al., 1993; Mutasa et al., 1994).

At the start of the work described in the present thesis, taking into consideration the facilities at and aims of a practice oriented research station, I chose to study the possibilities of obtaining quantitative information on the amount of BNYVV inoculum in soil by means of a bioassay, rather than trying to develop sophisticated methods for detection of virus directly in the soil. Besides, a bioassay allows the simultaneous study of virus and vector.

Serial dilution of a soil sample and assessment by bioassay of the presence of the pathogen in aliquots of the different dilutions permits an estimation of the most probable number (MPN; Cochran, 1950) of infective units of the pathogen in the original sample. In this way the density of infective propagules is determined, which is designated the inoculum potential (Pfender et al., 1982).

In the quantitative bioassay developed for 'rhizomania-infested' soil (Chapter 2), serial dilutions of the soil were used, as described before. The method permitted the assessment of infective inoculum densities of both *P. betae* and BNYVV in soil. The MPN of BNYVV can be interpreted as a measure of the population density of viruliferous *P. betae*. The ratio of the MPNs of BNYVV and *P. betae* is an estimate of the viruliferous proportion of the population *P. betae*, on the assumptions that the rate of germination and infection is the same for viruliferous and non-viruliferous spores of *P. betae* and that for detectable infection of bait plants by *P. betae* or BNYVV the same number of non-viruliferous or viruliferous propagules is required. If for *P. betae* a single primary infection would suffice to detect its presence after a 6-week incubation, but for BNYVV multiple primary infection would be required to multiply to detectable levels, the virus content of the population would be underestimated.

Comparison of the assessment of virus and vector after 6 weeks with that after only 3 weeks showed that the MPN of BNYVV increased more than the MPN of *P. betae*. This result indicates that the soil volume in the pot was sufficiently explored after 3 weeks to enable the majority of infective propagules of *P. betae* to infect and multiply to detectable levels, whereas for the multiplication of the transmitted virus to detectable levels more time was needed (Chapter 3). This finding is corroborated by Henry et al. (1992), who compared the qualitative detection of BNYVV in soil by different bioassay procedures, and concluded that shortening the seedling bait test to less than 6 weeks led to loss of sensitivity. Extension of the duration of the assay to 12 or 18 weeks did not significantly increase the MPNs determined for BNYVV and did not affect the qualitative detection level (Chapter 3).

The dilution endpoint for infested soil samples was c. 10^{-4} to 10^{-5}, so five tenfold dilutions were made and assayed for every soil sample. Undiluted soil was not included in the bioassay. If one is not interested in non-viruliferous *P. betae* and no severe infestation is expected, dilution 10^{-5} can be omitted. For most samples to be assayed for BNYVV, three tenfold dilutions would suffice to distinguish low from high levels of infestation.

The precise nature of the infective units of vector and virus determined with the quantitative bioassay, is unknown. For *P. betae*, I estimated that one infective unit represented between 50 and 100 resting spore clusters in artificially infested sand (Chapter 3). This estimate corresponds with data of Fujisawa and Sugimoto (1977) and with the finding of Keskin (1964) and Dahm (1993) that 1% of plants became infected by *P. betae* when inoculated with single resting spore clusters. Age and size of the resting spores seem not to have large effects on the infectivity.

With naturally infested soil, storage conditions did, but storage period did not, affect germination of spores during the first 48 hours of exposure of plants (Chapter 4). It was demonstrated that even a short period of drying decreased infectivity. Treatment of soil affects the results of bioassays only when short periods of exposure are applied, because quantitative assessment of inoculum with the 6-week bioassay was affected neither by storage conditions nor by storage period.

In short, an adequate quantitative bioassay for BNYVV and *P. betae* in soil was developed, by means of which it was shown that even in soils under heavily diseased crops no more than 15% of the population of spores was viruliferous.

Disease incidence and yield in relation to inoculum potential of BNYVV

A major goal in epidemiology is to gain insight in the relationship between inoculum potential, disease incidence and yield or yield loss. In pot experiments, root weight of beet was found to decrease with increasing amounts of BNYVV-infested soil in a soil mixture (Kanzawa, 1974; Bürcky et al., 1986; Chapter 3 in this thesis). In a review on the epidemiology and management of BNYVV, Schlösser (1988) mentioned that 'there are no detailed studies on the quality and quantity of inoculum required to initiate infection'. The field experiment described in Chapter 5 was the first to provide information on the inoculum density - yield relationship in the field. Different inoculum levels were created by applying small amounts of infested soil to field plots. In the first year after infestation, sugar content and quality parameters as sodium and α-amino nitrogen content were already affected at these infestation levels which could hardly be detected by bioassay. It was confirmed that sugar content is a sensitive parameter for rhizomania disease and so is sodium content (Heijbroek, 1989). It was demonstrated

that precise empirical threshold values based on these quality parameters (Wieninger and Rössner, 1983; Pollach, 1984; Heijbroek, 1989) have limited validity as indicators of rhizomania.

In the second year after infestation, when inoculum was readily detectable, a non-linear relationship between the MPNs of BNYVV (viruliferous *P. betae*) and yield parameters was found. The empirical relationship could be described by logistic and Gompertz equations. This approach in relating inoculum level to yield demonstrated that even though the vector has multiple secondary infection cycles, initial inoculum and primary infection were determining factors for disease incidence and severity under the prevailing environmental conditions, whether irrigated or non-irrigated. The relationship applied to a situation where a) inoculum had evenly been spread over the plot area and b) soil temperatures reached 15°C soon after sowing; it could be established because a wide range of inoculum levels was present. The area available for the field experiment described in Chapter 5 was not large enough to include a BNYVV-resistant cultivar for comparison of its response with that of the susceptible one. In cultivar trials, it was found that at low infestations where the inoculum density - yield curve is approximately horizontal (Chapter 5), yield of BNYVV-susceptible cultivars was equal or higher than that of partially resistant cultivars. Susceptible cultivars have a higher yield potential and their root weight was not affected but sugar content was decreased (Tuitert et al., unpublished).

In the field trial on relationships between inoculum present in soil, disease incidence and yield, a high soil sampling frequency was applied to ensure a representative sample. Such a sampling frequency would not be feasible for large scale practical application. On a field scale, the pattern of inoculum in the field and the sampling frequency determine the representativeness of the sample. For five cultivar trials at different locations, the mean MPNs of BNYVV of eight intensively sampled (1 core m^{-2}) plots per trial, together representing about 10% of the area of the trial field, were found to be linearly correlated with the relative yield of a susceptible versus a partially resistant cultivar (Tuitert et al, unpublished).

The MPN determined in a compound sample from a large area might result from soil cores which all contain approximately the same inoculum density or it might result from one, or a few, cores with high inoculum densities, taken from highly infested patches, combined with a majority of non-infested cores. Obviously, yield will be different in a situation with a moderate overall infestation or with highly infested patches in a non- or lightly infested field. Model studies by simulating patches of different sizes and inoculum levels combined with different sampling frequencies, could serve as a basis for the choice of the soil sampling strategy. When more data are available on the relationship between inoculum potential of BNYVV and yield of sugar beet in naturally infested fields under different environmental conditions, these model studies could provide information on the potential yield loss of a susceptible cultivar inferred from the MPN determined in the sample. Such an approach was taken by Schomaker and Been (1992) to evaluate sampling strategies for potato cyst nematodes. Theoretical probabilities of detection of circular patches with different diameters were calculated by Büttner and Bürcky (1990) and Asher and Henry (1993), but spatial distribution of BNYVV and sampling frequency were not extensively investigated. Besides, only the probability of the presence of inoculum in the sample was investigated and not the inoculum density.

As long as cultivars that are partially resistant to BNYVV have a lower yield potential under conditions with low or no infestation, the possibility of a yield

prediction will be interesting. With a MPN resulting from a patchy distribution, the yield of a susceptible cultivar might still be higher than that of a partially resistant one, whereas with an evenly dispersed inoculum this will be the reverse. On the assumptions that a) sugar yield of a susceptible cultivar on a severely infested field is 3 ton ha^{-1}, which is 30% of that of a partially resistant cultivar under these circumstances and b) yield of the susceptible cultivar under disease-free conditions is 13 ton ha^{-1}, which is 10% higher than the partially resistant cultivar; theoretically, yield of the susceptible cultivar on a field would be higher than that of the resistant cultivar when the severely infested area would not exceed 14% of the area of field.

The small area cropped with resistant cultivars by Dutch growers in 1992 (c. 850 ha), compared to the extent of the area suspected to be BNYVV-infested (Heijbroek, personal communication) shows a.o. that for many farmers the risk of damage was counteracted by the expected higher yield of susceptible cultivars when severity is low or when the infested area is thought to be limited. The severe manifestation of rhizomania in 1992, and the extra efforts of the sugar companies and the IRS in promoting the use of partially resistant cultivars, may have contributed to the increased area being cropped with these cultivars in 1993 (c. 2700 ha), which was still only 10% of the estimated infested area.

Disease severity or yield loss at different inoculum levels is strongly influenced by environmental factors. Under moist conditions, multiplication of *P. betae* is enhanced. Even brief intermittent rains can stimulate zoospore release and subsequent infection (De Heij, 1991). Enhancement of primary and secondary infection of plants by viruliferous *P. betae* will lead to an increase in BNYVV-content of the roots and to an increase in damage. It was demonstrated experimentally that the application of drip irrigation can aggravate the severity of BNYVV-infection in the field (Chapter 5).

Differences in soil type might affect the inoculum level - disease relationship, possibly largely because of different physical properties of the soil. Badly draining soils will have longer periods of optimal soil moisture conditions for zoospore dispersal and infection. Soils that warm up slowly in spring, may lead to a reduction in severity. As for the pH, acidic conditions (pH<5) inhibit rhizomania development because *P. betae* is negatively affected (Abe, 1974; 1987). These conditions are also unfavourable for sugar beet cultivation.

The time of primary infection of beets in spring will depend mainly on soil temperature. The minimum temperature of infection of beet roots by *P. betae* is between 10 and 12 °C (Abe, 1974; Asher and Blunt, 1987; De Heij, 1991). Onset of infection was later and rate of fungal development was lower at 15 °C than at 20-25 °C (Blunt et al., 1991). Optimum temperatures for virus multiplication in beet roots have not been determined in the absence of the vector. For plants growing in infested soil, the highest virus content was found at 25 °C (Horak and Schlösser, 1980). Symptoms of rhizomania are more severe and yield loss greater when infection occurs at an earlier growth stage of the plant (Abe, 1974). In the three years of the field experiment described in Chapter 5, soils warmed rather rapidly in spring.

In a recent review on rhizomania it was mentioned that 'the actual relationship between inoculum density and disease severity has never been critically established' (Asher, 1993). The present work is an attempt to contribute to filling this gap in the knowledge of the epidemiology of rhizomania.

Dynamics of inoculum

Inoculum is defined as 'the substance, generally a pathogen, used for inoculating' (Hawksworth et al., 1983). In the case of rhizomania disease, inoculum could be read as BNYVV particles, viruliferous zoospores or viruliferous resting spores of *P. betae*. Although the motile zoospores might literally represent the most dynamic stage in the life cycle of the vector, the dynamics of or changes in the infective population of viruliferous or non-viruliferous resting spores was the subject of study in Chapter 6.

A density-dependant multiplication was found (discussed in Chapter 6), both in the field for viruliferous *P. betae* (Chapter 6) as well as for non-viruliferous *P. betae* in growth chamber experiments (Chapter 8). The negative linear relationship between $\log_{10}$-transformed inoculum potential of BNYVV and $\log_{10}$ of the multiplication ratio, for the range of inoculum potentials available (Chapter 6), indicates a proportional decrease of multiplication. At the lower side of the range, the detection threshold of the bioassay does not allow detection of very low MPNs, which would have multiplication ratios higher than those found by extrapolation of the linear relationship (compare the ratios in 1988). A decrease of BNYVV inoculum at very high inoculum levels, as would be suggested by extrapolation of the fitted line, is not likely. It is, however, not known if a lack of infection sites on severely infected roots might prevent infection by so many zoospores that loss of resting spores by germination is no longer compensated for by the gain of new resting spores in the infected roots. The high BNYVV-content in the roots might also reduce the number of resting spores formed, as was observed by Schlösser (1990).

The design of the field experiment was aimed at limiting interplot interference by contamination. Subplots were not randomized, but the direction of plots with increasing inoculum levels was alternated. The non-infested plots were always at the far end or at the beginning of a strip, and thus were at risk of contamination from outside. Indeed, contamination of these plots, especially at the front, occurred, but it remained limited in the first two years. 'Positive interplot interference' (Campbell and Madden, 1990), by dispersal of inoculum from plots with a high level of inoculum to neighbours with a lower level cannot be fully discounted, but all activities (tillage, sowing, sampling) were performed so as to limit its occurrence (Chapter 6).

Extra moist conditions did cause a relatively small enhancement of multiplication of viruliferous inoculum in the first year of the trial (Chapter 6), but not in the second year. The response of resting spores to wetting, by rainfall or irrigation, does not have to be instantaneous (Chapter 4). Spores will need time for hydration. The duration of hydration depends, among others, on the prior conditions of the spore in the soil (Chapter 4). It might occur that, during the time it takes some spores to hydrate, soil moisture conditions have changed and no longer favour zoospore release with subsequent dispersal and infection.

It was found that growing beet for one season can be sufficient to cause a rapid increase of viruliferous inoculum from hardly detectable levels to detectable and damaging levels (Chapters 5 and 6). The ten-thousandfold multiplication ratios of BNYVV in the first cropping year were estimated using calculated initial densities for 1988. Multiplication ratios during the second beet crop, based on initial and final densities (1989 and 1990) estimated by the most probable number method, ranged from about 70 to 3. The lower multiplication in the second year might have been due to the level of viruliferous inoculum already present.

Starting from a very low population density, the total population of *P. betae* was also

estimated to have increased ten-thousandfold during two consecutive beet crops (Chapter 6). In view of the high multiplication ratio of *P. betae* estimated on the basis of the numbers of resting spores present in roots of 12-week-old plants growing at even higher inoculum densities than in the field (Chapter 8), this ratio does not seem unrealistic.

The rapid multiplication of inoculum of BNYVV from levels at which no symptoms were observed to levels with high disease severity in the next crop could explain why in a year with favourable conditions for rhizomania (e.g. 1992) high yield losses were recorded on fields considered to be non-infested. In practice, the usual explanation was that recent contaminations were responsible for the observed yield losses. Our results, however, show that contaminations need not to be recent since fast multiplication of inoculum occurs when conditions are favourable, and low levels may have been present before the previous beet crop.

Horizontal spread of BNYVV in soil

So far, temporal aspects of epidemic development of rhizomania were discussed. For an epidemic to develop, initial inoculum has to be present, which implies that dispersal of inoculum has taken place. Knowledge of distances of spread of the disease is required to interpret an epidemic. Some spatial aspects of rhizomania disease were studied; dispersal of inoculum and spread of disease (Chapter 7).

Dispersal of inoculum. Autonomous movement of zoospores through soil covers only small distances. In a hydroponic culture of beet, Dahm & Buchenauer (1993) observed that zoospores of *P. betae* attached to the root near the point of release rather than moving large distances before attachment. Distances of spread might be increased by passive transport of zoospores by soil water currents. Extension of infected roots will also result in spread of the virus: first, by recurrent infection of the roots by viruliferous zoospores; second, through acquisition of virus, translocated in the roots, by non-viruliferous vector propagules along the extending roots. In the field, viruliferous inoculum was found at 20-25 cm distance from point sources consisting of infected plants at the end of one cropping season. Root growth from healthy plants towards a point source of infested soil appeared to have contributed to dispersal of inoculum to a distance of 20 cm, as in fallow plots inoculum was not detected at this distance from the inoculum source.

The largest contribution to dispersal of inoculum within a field is by displacement of infested soil rather than by processes in the growing crop itself. Agricultural implements, especially harvesting equipment, can spread adhering soil over considerable distances. The impact of this spread was shown by the results in Chapter 7, where a disease gradient was found from 2 to 8 m distance from an infested plot in the second crop after infestation, whereas the whole area was completely diseased in the third year. Harveson and Rush (personal communication) detected BNYVV in soil samples taken at 23 m from a point source area of infestation after mechanical harvesting and seedbed preparation had taken place.

There are various ways in which infested soil or other material can be spread (Schlösser, 1988). The results of Chapters 5 and 6 indicated the potential hazards of the introduction of small amounts of infested soil to a field. The practical relevance of the chosen amounts was illustrated in Chapter 5. Phytosanitary measures to reduce the risk of spreading disease between fields, a.o. by measures to avoid or reduce the amounts of infested soil introduced into fields, have been advocated in several countries (Cariolle,

1987). The actual measures taken will depend on feasibility and relative effectiveness (Hofmeester, 1991).

Spread of disease. Virus-infected target plants were detected at very low incidence at 25 cm distance from a point source of inoculum and not at all at larger distances. The inference was made that increased incidence of disease in the plant population depends on infection from viruliferous resting spores of the vector in the soil, with subsequent re-infection of the roots, rather than on plant-to-plant spread of secondary zoospores (Chapter 7). As roots of neighbouring plants intermingle in the course of the growing season, plant-to-plant spread is expected to occur, but too late in the season to contribute to the observed development of the epidemic. The hypothesis remains to be tested in plots sown as in commercial practice with sources of infection within the row, but it is supported by the dependency of disease incidence and yield on inoculum levels in soil (Chapter 5). Randomness of infection in an randomly infested area would provide additional circumstantial evidence (Campbell and Madden, 1990). Recently Harveson and Rush (1993) reported that 'very little movement of BNYVV was detected outside of the inoculated areas', but, as their nearest sampling distance appeared to be 4.5 m (Harveson and Rush, personal communication), their observation cannot be used as a confirmation of the suggested minor role of plant-to-plant spread in the epidemic.

Effect of virus-resistant cultivars on build-up of BNYVV inoculum in soil

The most promising and the only practical way to control rhizomania disease of sugar beet is by growing BNYVV-resistant cultivars. Breeding for resistance to rhizomania disease has yielded a number of cultivars that are partially resistant to the virus, but not to the vector. Yield parameters and virus content of these cultivars were reported several times (references in Chapter 8), but their effect on soil inoculum was not considered. This effect is of major importance in view of the longevity of the vector and the persistence of the virus under field conditions. The hypothesis was tested that acquisition of virus by the vector is reduced in virus-resistant plants and that, consequently, virus-resistant cultivars retard the build-up of soil-borne inoculum of the virus (Chapter 8). It was indeed found that in partially resistant plants with a low virus content in the root less viruliferous spores were formed than in susceptible plants with a high virus content. The total number of resting spores formed in the roots did not show a difference between the two cultivars. Up to 50% of freshly isolated resting spores from roots of a susceptible cultivar contained BNYVV, whereas 15% at most of populations of infective units of *P. betae* in field soil was found to contain the virus (Chapters 2, 5, 8).

In the field, differences in build-up of inoculum of BNYVV between a susceptible and partially resistant cultivar could hardly be found by bioassay (Chapter 8). Various explanations may be offered (Chapter 8), e.g. the heterogeneity of the virus content in partially resistant plants (Paul et al., 1992) and the possibility that only a small proportion of viruliferous resting spores germinate during the season. At the five locations sampled, the increase in viruliferous inoculum was small compared to the increases found in the artificially infested field mentioned in Chapter 6. Differences in spatial distribution of inoculum between naturally and artificially infested fields and in environmental conditions may be some of the factors responsible for this difference.

A higher level of virus resistance of the host plant will have a larger effect on the viruliferous inoculum in the field soil. As a measurable decline in levels of BNYVV in soil will also depend on the proportion of viruliferous spores that germinate during the

season, the effects of (partially) resistant cultivars are perhaps only measurable when these cultivars are grown for more than one year on the same site. To ensure that virus-resistant cultivars will continue to perform well in infested fields, it is important that they do not increase the inoculum level in the soil. When the transmission ratio between resting spores from a susceptible and a resistant cultivar (Chapter 8) would be reflected in the ratio of inoculum levels under field conditions, yield of a following beet crop could be higher after a resistant than after a susceptible cultivar (inferred from yields at different inoculum levels in the field experiment described in Chapter 5).

Resistance to the vector might contribute to a further delay in build-up of inoculum or a gradual elimination of inoculum of BNYVV, provided that the induction of germination of resting spores by the resistant roots is not affected. A reduction in the build-up of viruliferous inoculum will contribute to the durability of disease resistance.

Conclusions

A practical bioassay method was developed that allowed quantitative assessment of infective populations of *P. betae* and BNYVV (viruliferous *P. betae*) in soil. By assessing the numbers of infected plants in serial dilutions of soil, the MPN of infective units of non-viruliferous or viruliferous vector could be estimated. Of the fungal resting spore clusters in soil 15% at most contained BNYVV. A 6-week duration of the bioassay gave adequate results, and treatments of soil prior to assessment only affected infectivity during the first 48 hours of exposure of bait plants. One infective unit of *P. betae*, as measured by the MPN method, represented 50 to 100 resting spore clusters in artificially infested soil. Of fresh resting spore clusters up to 50% contained virus.

Small amounts of infested soil introduced into a non-infested field were shown to affect sugar yield in the first beet crop grown on the field, thus demonstrating the ease with which small amounts of inoculum can be transferred in soil, and emphasizing the importance of phytosanitary measures. In the second year after infestation, yield losses up to 66% occurred. Using the bioassay, the relationship between inoculum potential of BNYVV in soil, disease incidence and yield was demonstrated under field conditions for the first time. It was shown that extra moist conditions can aggravate disease severity. Quantification of viruliferous and non-viruliferous vector in soil demonstrated the rapid multiplication of both; from hardly detectable levels at which the beet crop appeared healthy, to levels at which disease severity was high.

Results of experiments on horizontal spread of BNYVV in soil contributed to the understanding of the epidemiology of the disease and showed the impact of displacement of infested soil by agricultural implements.

When cultivars have a high level of resistance against BNYVV, they may delay the build-up of inoculum. Resistance to the vector will help to reduce the multiplication of inoculum in soil. A delayed build-up or a gradual elimination of the virus in soil are essential contributions to the durability of disease resistance. Resistant cultivars will be more widely used when the continuing efforts of the breeding companies have resulted in cultivars with higher yield potentials under disease-free conditions and higher levels of resistance.

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Summary

Summary

Rhizomania disease of sugar beet is caused by beet necrotic yellow vein virus (BNYVV). Rhizomania ('root madness') can severely reduce sugar yield of beet. Besides, the presence of inoculum in the soil threatens the export of planting material, like seed potatoes, grown in that soil, because of import constraints in countries considered to be free of the disease. The fungus *Polymyxa betae*, an obligate parasite, is the soil-borne vector of BNYVV. The longevity of the fungal resting spores in soil and the persistence of virus particles in the spores restrict the possibilities to control the disease. Breeding for resistance is considered to be the most promising way of control. Quantitative epidemiology of the disease received little attention.

The research described in this thesis is introduced in *Chapter 1*. Basic epidemiological questions were addressed, such as a) the amount of inoculum necessary to initiate disease and the development of disease at different inoculum levels, b) the polyetic dynamics of inoculum in soil, c) the dispersal of inoculum and spread of disease and d) the effect of virus resistance of beet on the viruliferous vector population.

Quantitative epidemiological studies depend on adequate methods of detection and quantification of inoculum in soil. A quantitative bioassay method was developed that enabled estimation of densities of both virus (viruliferous vector) and vector in soil (*Chapter 2*). The most probable number (MPN) of infective units of *P. betae* with or without virus was estimated from the incidence of infected seedlings in a dilution series. The estimates obtained were designated as inoculum potentials of virus and vector. MPNs ml^{-1} assessed for samples from two fields in the Netherlands were 48 for *P. betae* with 7.1 for BNYVV and 16 for *P. betae* with 1.6 for BNYVV, respectively. So in these soils 10-15% of the infective population of *P. betae* was viruliferous. Differences in conditions during storage of soil samples for 28 months - dry at room temperature or moist and cool - did not lead to differences in inoculum potentials.

For artificially infested sand, it was estimated that one infective unit of *P. betae* in the quantitative bioassay represented between 50 and 100 resting spore clusters, as the average recovery was 1.7% (*Chapter 3*). Assessment of vector and virus after 6 weeks yielded 3- and 16-fold higher MPNs, respectively, than when determined after 3 weeks. A 6-week duration of the bioassay was adequate for detection of BNYVV. Neither prolongation of the duration from 6 to 18 weeks, nor a second and third 6-week replant resulted in a lower dilution endpoint of BNYVV in naturally infested soil. Estimates of inoculum potential of BNYVV were not significantly increased when incubation was extended over 6 weeks. Fresh weights of tap roots from bioassay plants progressively decreased with increasing amounts of inoculum, but weight of lateral roots was hardly affected.

The effects of storage conditions and pre-treatments of infested soil on infectivity of resting spores of *P. betae* were determined by assessing infection of bait plants exposed to the soil for 12 to 72 h (*Chapter 4*). Air-dry storage of soil at room temperature resulted in a delayed onset of germination of resting spores compared to germination in soil stored under moist and cool conditions. Germination of resting spores and subsequent infection of bait plants in flooded infested soil required over 12 h. However, when soil was prewetted for 24 h before exposing the bait plants, germination, infection and transmission of virus were accomplished within 12 h, but only with the moist soil. The higher infectivity of spores stored under moist conditions was confirmed by

experiments using resting spores isolated from roots. It was concluded that storage conditions and pre-treatments of soil influence the results of bioassays for detection of rhizomania only when short baiting periods are applied.

A field experiment was set up in 1988 to study the development of rhizomania disease in a susceptible sugar beet cultivar at different inoculum levels of BNYVV in soil (*Chapter 5*). The five inoculum levels were created by application of different amounts of BNYVV-infested soil to field plots free of BNYVV. A drip irrigation treatment was included to investigate the influence of soil moisture on disease. The cultivar 'Regina' was grown for three years consecutively. Disease incidence, yield and quality parameters were determined. In the first year, root symptoms were not observed, but at harvest BNYVV-infected plants were detected by ELISA in low numbers at all inoculum levels. Root weight at harvest was not affected, but sugar content decreased with increasing inoculum level, leading to a reduction in sugar yield of 10% at the highest inoculum level. In 1989, root symptoms were observed already in June. Disease incidence showed an increase with increasing inoculum level, up to c. 100% at the highest inoculum level, and increased during the season. Symptoms of BNYVV-infected plants were more severe in plots with higher inoculum levels. Root weight and sugar content decreased progressively with increasing inoculum level, resulting in sugar yield reductions of 11 to 66% compared to the non-infested plots. The relationship between the inoculum potentials of the plots and yield parameters could be described by Gompertz or logistic curves. In the third year, the whole field was heavily diseased, and also the non-infested control plots were contaminated. Yields were low, but still showed a decrease with increasing inoculum level from 6 to 4 ton ha^{-1} in the non-irrigated plots. For the irrigated plots the overall mean sugar yield was only 3323 kg ha^{-1}. In conclusion, the results showed that a) introduction of small amounts of infested soil into a field can cause yield reductions in the first and more severely in the second beet crop grown and b) sugar content and additional quality parameters (content of sodium and amino nitrogen) react to very low inoculum densities.

In the same field experiment, the dynamics of inoculum of BNYVV and *P. betae* were studied during the first two years after infestation (*Chapter 6*). In soil samples taken after the first sugar beet crop, differences between the plots to which initially different amounts of inoculum were applied could be detected by the quantitative bioassay. Both after 1 and after 2 years, the initial inoculum level of 1988 had a significant effect on log-transformed MPNs of BNYVV determined. Log-transformed MPNs of 1990 and 1989 showed a positive linear correlation, but multiplication of BNYVV decreased at higher inoculum levels. The first sugar beet crop resulted in a ten-thousandfold multiplication of inoculum of BNYVV (viruliferous *P. betae*), whereas after the second one the maximum increase was c. 70-fold. Irrigation during one or two seasons resulted in approximately threefold higher MPNs than without irrigation. The total *P. betae* population was also higher after growing two irrigated crops than after growing two non-irrigated ones. The rapid increase of inoculum highlights the hazards of small amounts of infested soil and emphasizes the importance of measures to reduce spread of infested soil.

Spatial aspects of rhizomania disease were studied in greenhouse and field experiments (*Chapter 7*). In the greenhouse, horizontal spread of BNYVV by means of viruliferous zoospores of *P. betae* was less than 5 cm. In the field, active dispersal of the vector, in combination with root growth from and towards point sources of inoculum, contributed little to horizontal dispersal of viruliferous inoculum and spread of disease during the season. In the second beet crop after application of inoculum to entire field

plots, more BNYVV-infected plants were detected at 2 m than at 8 m distance from the infested plots in the direction of tillage. In the third year, disease incidence at 8 m was high and equivalent to that at 2 m, thus showing the effects of soil displacement by machinery.

Sugar beet cultivars with partial resistance against BNYVV have been commercially released and are widely grown in BNYVV-infested areas in Europe. The hypothesis was tested that growing virus-resistant beet cultivars would reduce virus content of the vector population (*Chapter 8*). The total number of resting spore clusters of *P. betae* formed in roots of a partially resistant and a susceptible cultivar did not differ when assessed 6 and 12 weeks after inoculation with viruliferous resting spores. However, in partially resistant plants with a low virus content in the roots, the population of resting spores formed was less viruliferous than that in susceptible plants with a high virus content. Consequently, growing a resistant cultivar can be expected to delay the build-up of virus inoculum in soil. In a trial field sampled in 1991, the inoculum potential of BNYVV (viruliferous *P. betae*) in soil was indeed lower after growing a partially resistant cultivar than after growing a susceptible one. On the other hand, in four sites sampled in 1990, inoculum potential in soil was hardly increased by growing sugar beet and was not significantly affected by the cultivar grown.

A comprehensive discussion of the results presented in this thesis was given in *Chapter 9*. The main points are the following. An adequate method was developed to quantify the infective populations of viruliferous and non-viruliferous *P. betae* in soil. This 'MPN-method' allowed quantitative epidemiological studies on the dynamics of inoculum and on the relationships between soil inoculum and disease incidence and yield. The risk of spread of infested soil was demonstrated. Thus the importance of phytosanitary measures to prevent displacement of infested soil was emphasized. A major role of soil-borne inoculum in determining disease incidence and yield was shown. The relationships found could serve as circumstantial evidence for the hypothesis, based on the limited spread of disease found in experiments with point sources of inoculum, that the incidence of BNYVV in the field depends on infection from resting spores in the soil, with subsequent re-infection of the roots, rather than on plant-to-plant spread by secondary zoospores. It was shown that low levels of inoculum can rapidly multiply to damaging levels. An inverse relationship between multiplication ratio and inoculum level was demonstrated. Resistance of sugar beet to BNYVV can decrease virus content of newly formed resting spores and eventually reduce the viruliferous inoculum in the field. A reduction in build-up of inoculum of BNYVV will contribute to the durability of disease resistance.

In short, it was demonstrated that inoculum of rhizomania disease of sugar beet can rapidly build up to damaging levels and that virus-resistance of beet can have long-term beneficial effects by delaying the build-up of inoculum. Higher levels of virus resistance than the current partial resistance are required for significant reductions of viruliferous inoculum in the field.

Samenvatting

Samenvatting

Rhizomanie ('wortelgekte') bij suikerbiet wordt veroorzaakt door het bietenrhizomanievirus (BNYVV). De ziekte kan grote verliezen aan suikeropbrengst veroorzaken. Bovendien vormt de aanwezigheid van inoculum in de grond een bedreiging voor de teelt van bijvoorbeeld pootaardappelen voor de export, vanwege quarantaine maatregelen in landen waar de ziekte nog niet of in geringe mate voorkomt. Het virus wordt overgedragen door *Polymyxa betae,* een obligaat parasitair levende wortelschimmel. De lange levensduur van de rustsporen van de schimmel en de persistentie van het virus in deze sporen beperken de mogelijkheden van bestrijding van de ziekte. De ontwikkeling van resistente cultivars wordt beschouwd als de beste wijze om schade door rhizomanie tegen te gaan. Over de kwantitatief epidemiologische aspecten van de ziekte was weinig bekend.

Het in dit proefschrift beschreven onderzoek, dat werd ingeleid in *Hoofdstuk 1*, was gericht op het verkrijgen van inzicht in de epidemiologie van rhizomanie in suikerbiet. Er werd aandacht geschonken aan fundamentele aspecten in de epidemiologie van de ziekte: a) de hoeveelheid inoculum nodig om ziekte te veroorzaken en de ontwikkeling van de ziekte bij verschillende inoculumdichtheden; b) de polyetische dynamiek van inoculum in de grond onder invloed van een waardgewas; c) de verspreiding van inoculum en ziekte; d) het effect van virusresistentie van het waardgewas op de virusbevattende vectorpopulatie.

Een eerste vereiste voor het uitvoeren van kwantitatief epidemiologisch onderzoek is de beschikking over een adequate methode om inoculum in grond te detecteren en te kwantificeren. Daarom werd een kwantitieve biotoets ontwikkeld waarmee de populatiedichtheden van vector en virus (virusbevattende vector) in grond geschat konden worden, in termen van 'meest waarschijnlijke aantal infectieuze eenheden' (MPN-waarden) per ml grond (*Hoofdstuk 2*). In grondmonsters van twee met rhizomanie besmette percelen werden inoculumdichtheden geschat van 7.1 en 1.6 ml^{-1} voor BNYVV, met 48 en 16 ml^{-1} voor *P. betae*. In deze gronden bleek 10-15% van de infectieuze vectorpopulatie het virus te bevatten. Een verschil in omstandigheden tijdens bewaring van grondmonsters gedurende 28 maanden leidde niet tot een verschil in schatting van de inoculumdichtheid.

Bij toetsing van kunstmatig besmet zand was de geschatte inoculumdichtheid voor *P. betae* gemiddeld 1.7% van de hoeveelheid toegediende rustsporenclusters. Eén infectieuze eenheid van *P. betae* vertegenwoordigde dus 50-100 rustsporenclusters (*Hoofdstuk 3*). De MPN-waarden voor virus en vector na 6 weken waren 3- en 16-maal hoger dan de waarden bepaald na 3 weken. Een verlenging van de incubatieduur van de biotoets na 6 weken niet leidde tot een lagere detectiegrens en evenmin tot een significant hogere MPN-waarde voor BNYVV. De gewichten van de penwortels van de biotoetsplanten namen af bij toenemende hoeveelheid besmette grond in het substraat, het gewicht van de laterale worteltjes werd nauwelijks beïnvloed.

De effecten van verschillende behandelingen van grond op het infectievermogen van rustsporen van *P. betae* werd bepaald door bietekiemplanten gedurende korte tijd te incuberen in een suspensie van de grond (*Hoofdstuk 4*). Wanneer grond droog bij kamertemperatuur was bewaard werd een vertraagde kieming van rustsporen waargenomen ten opzichte van grond die vochtig en koel was bewaard. Een tijdsduur langer dan 12 uur was vereist voor kieming van rustsporen in grond en infectie van de blootgestelde kiemplanten. Wanneer daarentegen de grond gedurende 24 uur nat gezet

werd voorafgaand aan de incubatie van de kiemplanten, bleek kieming, infectie en overdracht van BNYVV op te treden binnen 12 uur, maar alleen bij de vochtig bewaarde grond. Experimenten met uit wortels geïsoleerde rustsporen bevestigden het grotere infectievermogen van sporen die onder vochtige condities werden bewaard. De resultaten wezen uit dat condities gedurende bewaring van grondmonsters de resultaten van biotoetsen alleen beïnvloeden wanneer een korte incubatieduur wordt toegepast.

In 1988 werd een veldproef opgezet om de ontwikkeling van rhizomanie bij verschillende aanvangsdichtheden van BNYVV in de grond te bestuderen (*Hoofdstuk 5*). Er werden vijf besmettingsniveaus gecreëerd door verschillende hoeveelheden besmette grond toe te dienen aan nog onbesmette veldjes. De invloed van extra vochtige condities op de ontwikkeling van de ziekte werd bestudeerd door een behandeling met druppelirrigatie op te nemen. Gedurende drie achtereenvolgende jaren werd de gevoelige cultivar 'Regina' geteeld en werden ziekte-incidentie, opbrengst en kwaliteitsparameters bepaald. In het eerste jaar werden geen symptomen op de wortels waargenomen, maar bij de oogst bleek bij alle besmettingsniveaus in enkele planten virus voor te komen. Dit werd aangetoond met de serologische detectiemethode 'ELISA' (enzyme-linked immunosorbent assay). Er waren geen effecten op het wortelgewicht, maar het suikergehalte nam af bij toenemend besmettingsniveau, wat resulteerde in een reductie van de suikeropbrengst met 10% bij het hoogste besmettingsniveau. In 1989 werden in juni al planten met symptomen op de wortels aangetroffen. De incidentie van zieke planten nam toe gedurende het seizoen en was hoger bij hogere besmettingsniveaus, tot c. 100% bij het hoogste. De mate van symptoomexpressie van viruspositieve planten was sterker bij planten uit de hoogste besmettingsniveaus. Zowel het wortelgewicht als het suikergehalte vertoonden een afname bij toenemende besmetting, resulterend in suikeropbrengstreducties van 11 tot 66% in vergelijking met de onbesmette controle. De relatie tussen het gekwantificeerde inoculum in de grond en de opbrengstparameters kon beschreven worden met Gompertz of logistische curves. In het derde jaar was het gehele gewas ziek, de onbesmette controles waren ook besmet geraakt. De opbrengsten waren laag, maar de niet-geïrrigeerde veldjes vertoonden nog steeds een afname bij toenemend initiëel besmettingsniveau, van 6 tot 4 ton ha^{-1}. De gedurende 3 jaar geïrrigeerde veldjes hadden een gemiddelde suikeropbrengst van slechts 3323 kg ha^{-1}. Uit de resultaten kan men concluderen dat a) introductie van een zeer geringe hoeveelheid besmette grond in een perceel tot opbrengstreducties kan leiden meteen in het eerste bietengewas en in sterkere mate in het tweede gewas, en b) suikergehalte en additionele kwaliteitsparameters (gehaltes natrium en amino stikstof) al reageren op zeer lage inoculumdichtheden.

In het hiervoor beschreven veldexperiment werden de veranderingen in inoculumdichtheid van BNYVV en *P. betae* bestudeerd gedurende de eerste twee jaar na besmetting (*Hoofdstuk 6*). In grondmonsters genomen na het eerste bietengewas konden met de kwantitatieve biotoets verschillen in inoculumdichtheid van BNYVV worden aangetoond tussen de veldjes met verschillende aanvangsbesmettingen. Zowel na 1 als na 2 jaar bleken de log-getransformeerde MPN-waarden van BNYVV significant beïnvloed te zijn door de aanvangsniveaus van besmetting. Log-getransformeerde MPNs van 1989 en 1990 (na 1x en 2x bieten) vertoonden een positieve lineaire correlatie. De vermenigvuldiging van BNYVV (virusbevattende *P. betae*) nam af bij toenemende inoculumdichtheid. Geschat werd dat het eerste bietengewas een tienduizendvoudige toename van inoculum van BNYVV (virusbevattende *P. betae*) veroorzaakte. Het tweede gewas zorgde voor hooguit nog een zeventigvoudige toename. In de gedurende 1 of 2 jaar geïrrigeerde veldjes waren de MPNs gemiddeld driemaal

hoger dan in de niet-geïrrigeerde veldjes. De totale populatie *P. betae* was ook hoger na twee jaren met extra vochtige condities. De aangetoonde snelle toename van het inoculum maakt de risico's zichtbaar die aan kleine hoeveelheden besmette grond kunnen kleven en benadrukt aldus het belang van bedrijfshygiënische maatregelen om verspreiding van besmette grond zoveel mogelijk te beperken.

In *Hoofdstuk 7* kwamen ruimtelijke aspecten van rhizomanie aan de orde. In een kasproef bleek dat de horizontale verplaatsing van BNYVV via virusbevattende zoosporen minder was dan 5 cm. Ook in het veld bleek actieve verplaatsing van de vector, in combinatie met wortelgroei vanuit of naar de puntbronnen van besmetting, slechts in geringe mate verantwoordelijk te zijn voor horizontale verspreiding van BNYVV inoculum en uitbreiding van de ziekte gedurende de teelt. Bij de tweede teelt na het verspreiden van inoculum over de gehele oppervlakte van veldjes, werden meer geïnfecteerde planten aangetoond op 2 m dan op 8 m afstand van deze veldjes in de richting waarin de mechanische grondbewerking was uitgevoerd. In het derde jaar was de ziekteincidentie op 8 m afstand hoog en gelijk aan die op 2 m, aldus de effecten tonend van grondverplaatsing door machines.

Een aantal cultivars met partiële resistentie tegen BNYVV zijn in de handel en worden op vele plaatsen in Europa ingezet. De hypothese werd getoetst dat resistentie van biet tegen BNYVV de populatie virusbevattende *P. betae* vermindert (*Hoofdstuk 8*). De totale aantallen rustsporenclusters van *P. betae* in wortels van een partieel resistente en een gevoelige cultivar bleken niet te verschillen, bij bepaling op 6 en 12 weken na inoculatie met virusbevattende rustsporen. Echter, van de populatie rustsporen gevormd in partieel resistente planten met een laag virusgehalte in de wortels was een kleiner deel virusbevattend dan van de populatie sporen gevormd in vatbare planten met hoog virusgehalte. Bijgevolg is de verwachting dat een resistente cultivar de toename van inoculum in de grond vertraagt. In grondmonsters uit een proefveld in 1991 was de inoculumdichtheid van BNYVV na de teelt van een partieel resistente cultivar inderdaad lager dan die na een vatbare cultivar. Daarentegen werd op vier proefvelden in 1990 nauwelijks een toename in inoculum waargenomen; en had resistentie van de biet geen aantoonbare invloed op de dichtheid van virusbevattende *P. betae* na de teelt.

De inhoud van *Hoofdstuk 9* bestaat uit een algemene discussie van de in dit proefschrift beschreven resultaten. De belangrijkste punten kunnen als volgt worden samengevat. Er werd een biotoetsmethode ontwikkeld waarmee de besmetting van grond met BNYVV en *P. betae* gekwantificeerd kon worden. Deze 'MPN-methode' maakte het mogelijk om kwantitatief epidemiologisch onderzoek te doen naar de dynamiek van het inoculum en de relatie tussen inoculumdichtheid, ziekte-incidentie en opbrengst. De resultaten maakten duidelijk welke risico's verbonden zijn aan verspreiding van reeds kleine hoeveelheden besmette grond, en benadrukken aldus het belang van fytosanitaire maatregelen om verplaatsing van besmette grond te beperken. Er werd aangetoond dat de inoculumdichtheid in grond voor een belangrijk deel de ziekte-incidentie en opbrengst bepaalt. De gevonden relaties vormen een indirect bewijs voor een hypothese die gebaseerd werd op de beperkte uitbreiding van rhizomanie vanuit puntbesmettingen. Er werd gesteld dat de toename van ziekte-incidentie gedurende het seizoen meer wordt bepaald door het inoculum in de grond in de vorm van virusbevattende rustsporen, en door herinfectie van het eigen wortelstelsel, dan door verspreiding van plant tot plant via zoosporen. Uit de resultaten bleek verder dat kleine hoeveelheden inoculum zich zeer snel tot een schadelijk niveau kunnen vermenigvuldigen. Vermenigvuldiging van inoculum bleek dichtheidsafhankelijk te verlopen. De teelt van BNYVV-resistente cultivars kan de fractie virusbevattende

rustsporen binnen de nieuwgevormde populatie rustsporen verlagen en daarmee de hoeveelheid virus-inoculum dat in de grond terecht komt. Een geringere toename van inoculum in de grond, waaraan resistentie van biet tegen *P. betae* zou kunnen bijdragen, is van belang voor de duurzaamheid van (partieel) resistente rassen.

Kortom, er is aangetoond dat inoculum voor rhizomanie van suikerbiet zich zeer snel kan vermenigvuldigen tot schadelijke niveaus en dat virus-resistentie van suikerbiet de toename van inoculum kan vertragen. Een hoger en homogener niveau van virus-resistentie dan de huidige partiële resistentie is nodig voor een significante reductie van BNYVV inoculum in de grond.

Nawoord

In 1987 begon ik aan mijn driejarige contract als wetenschappelijk medewerker op het IRS om onder de brede titel 'epidemiologie en diagnostiek van rhizomanie' een bijdrage te leveren aan het gangbare onderzoek op dat instituut. Ik had wel de wens om te promoveren, maar verloor snel de illusie dat in het genoemde driejarig project te kunnen realiseren. Maar toen het project wat duidelijker vorm begon te krijgen en het contract met drie jaar werd verlengd, bood dat extra mogelijkheden voor onderzoek en stimuleerde dat daarenboven een extra inzet om te komen tot een zodanige verslaglegging dat het een proefschrift waardig zou kunnen zijn. Het resultaat ligt hier nu, en ik maak van de gelegenheid gebruik om een aantal mensen te bedanken voor hun bijdrage, in welke vorm dan ook, aan de uitvoering van mijn onderzoek en de realisering van dit boekje.

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Dat het uiteindelijk tot een promotie gekomen is, is zeker ook de verdienste van iemand die vanaf het begin in 1987 bleef geloven in die promotie; iemand die altijd bereid was tot een bespreking van de voortgang van het onderzoek, die een schouderklopje gaf wanneer dat nodig leek, en die als kritische en zorgvuldige lezer van mijn manuscripten gezorgd heeft voor o.a. een begrijpelijker taalgebruik (al is de inperking van mijn 'uitvoerigheid' nog niet volledig gelukt), een echte mentor dus: Gerrit Bollen. Ik prijs me gelukkig met zo'n co-promotor! Ik had daarnaast een uitstekende promotor: professor J.C. Zadoks. Ik ben hem zeer erkentelijk voor de kritische correctie van mijn manuscripten en stimulerende discussies over de beschreven resultaten.

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over met name de niet-lineaire regressie, waren onontbeerlijk en heb ik zeer op prijs gesteld. De leden van de studiegroep rhizomanie dank ik voor de goede contacten en overleg.

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Curriculum vitae

Gerrie Tuitert werd geboren op 17 december 1959 te Laren (Gld). In 1978 behaalde zij het Atheneumdiploma aan de Rijksscholengemeenschap te Lochem. In hetzelfde jaar begon zij met de studie Planteziektenkunde aan de Landbouwhogeschool (nu Landbouwuniversiteit) te Wageningen. Nadat zij in 1983 met lof het kandidaatsexamen behaalde, ging zij 10 maanden op stage voor de vakgroep Fytopathologie. Zij verrichtte mycologisch werk op de Brawijaya Universiteit te Malang in Indonesië en maakte een studiereis langs plantages en instituten op Java en Madura. Vervolgens werkte zij aan bodemgebonden pathogenen op het Plant Research Institute Burnley te Melbourne, Australië. De doctoraalstudie omvatte de hoofdvakken Fytopathologie en Theoretische Teeltkunde en het bijvak Nematologie. Gedurende deze tijd werd onderzoek verricht naar microbiële herkolonisatie en ontwikkeling van antagonisme tegen bodemgebonden pathogenen in gestoomde kasgrond, naar de waardplant-pathogeen interactie tussen *Cladosporium fulvum* en tomaat en naar de horizontale migratie van *Globodera pallida* in grond. Verder werd gewerkt aan de simulatie van worteldichtheid en wortelverdeling van aardappel in een gewasgroeimodel. Met het extra vak Pedagogiek en Didactiek werd de onderwijsbevoegdheid voor biologie verkregen. In januari 1987 werd het doctoraalexamen met lof behaald. Van 1987 tot 1993 was zij aangesteld als wetenschappelijk medewerker aan het Instituut voor Rationele Suikerproduktie te Bergen op Zoom. Resultaten van het aldaar verrichtte onderzoek zijn verwerkt in dit proefschrift. Vanaf juni 1993 is zij werkzaam als toegevoegd onderzoeker bij de vakgroep Fytopathologie van de Landbouwuniversiteit Wageningen.

Gerrie Tuitert was born on 17 December 1959 in Laren (Gld), the Netherlands. After finishing High School in 1978, she studied Plant Pathology at the Wageningen Agricultural University. She passed her BSc examination (with honours) in 1983. Thereafter, she had a 10-month practical training in phytopathology. She did mycological work at the Brawijaya University of Malang in Indonesia and made a study tour along plantations and institutes on Java and Madura. Next, she worked on soil-borne pathogens at the Plant Research Institute Burnley in Melbourne, Australia. She majored in Phytopathology, Theoretical Production Ecology and Nematology. The research subjects comprised microbial recolonization and development of antagonism against soil-borne pathogens in steamed greenhouse soil, the plant-pathogen interaction of *Cladosporium fulvum* and tomato and horizontal migration of *Globodera pallida* in soil. She simulated the density and spatial distribution of roots in a crop growth model of potato. By means of an extra topic Educational Science, she acquired the qualification for teaching biology. In January 1987, she obtained her 'Ingenieurs' or MSc degree (with honours). From 1987 to 1993, she had a research position at the Sugar Beet Research Institute at Bergen op Zoom, the Netherlands. Results of the work performed there are compiled in this PhD-thesis. In June 1993, she accepted a research position at the Department of Phytopathology of the Wageningen Agricultural University.

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List of publications

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