European Journal of Plant Pathology **100**: 201–220, 1994.

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

G. TUITERT[1,2], P.M.S. MUSTERS-VAN OORSCHOT[1] and W. HEIJBROEK[1]

[1] *Sugar Beet Research Institute, P.O. Box 32, 4600 AA Bergen op Zoom, The Netherlands;*
[2] *Present address: Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands*

Accepted 15 March 1994

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

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.

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 were 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 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 and 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

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 (× 1000) 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	A: 0.1, 1, 10, 100
(numbers ml^{-1} sand)			B: 1, 10, 100, 1000	B: 1, 10, 100, 1000
Assay of virus transmission				
Tube size (diameter × length, cm)			2 × 10	2.4 × 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 appliedd to test plants in order to assess virus transmission.

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 that 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, form 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 h) 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 and 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 (× 1000) 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, 2×10^3 and 1×10^6 resting spore clusters per pot (20 pots per inoculum level). Pots were placed randomly in a growth chamber at 22 °C during daytime (16 h) 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 mixed 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 winter in the beginning of the following year (Table 2). Each plot was sampled

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 × 7	01-05-90	29-01-91	7.3	3.5	6.3
NOPV90	1990	Nagele 'V'	6 × 7	02-05-90	07-03-91	7.3	4.5	7.6
ARN90	1990	Arnemuiden	6 × 7	01-05-90	30-01-91	7.3	2.2	9.5
NOPB90	1990	Nagele 'B'	6 × 7	02-05-90	22-03-91	7.2	4.5	7.9
THO91	1991	Tholen	3 × 16	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.

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].

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.

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).

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 tested. 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) × 100).

[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) × 100).

[e] MPN BNYVV divided by MPN *P. betae* (× 100).

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 4.1×10^3 (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 4.7×10^6 (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 2×10^3 and 1×10^6 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 5.2×10^3 per mg compared to 7.6×10^3 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 6.2×10^3 per mg root ($\log_{10} = 3.79 \pm 0.06$). The total number of spores formed per plant was 13.3×10^6 ($\log_{10} = 7.12 \pm 0.06$). The multiplication ratio was highest at the lowest inoculum level (Table 4).

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 and more so 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 diseases 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

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, $\log_{10}$ (abs × 1000)[b]		
	Density $\log_{10}$ n mg^{-1}	Multiplication ratio, $\log_{10}$	Tap root	Laterals	Tap root		Laterals
					R	S	
0	–	–	0.57 (3.69)	0.26 (1.80)	1.00 (9)	0.58 (4)	1.36 (22)
2×10^3	3.88 (7600)	3.90 (7850)	0.48 (3.01)	0.40 (2.52)	1.60 (39)	2.13 (132)	2.30 (196)
1×10^6	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.

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[a]				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

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

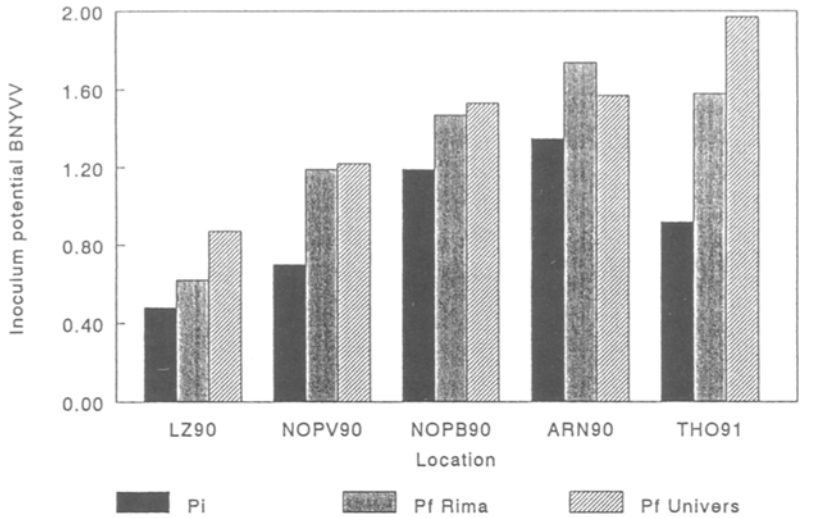

Fig. 1. The inoculum potential ($\log_{10}$ MPN + 1) of BNYVV in soil, before (Pi) and after (Pf) growing a partially resistant (Rima) and a susceptible sugar beet cultivar (Univers) at five locations. Locations and significances of cultivar effects are described in Tables 2 and 5, respectively.

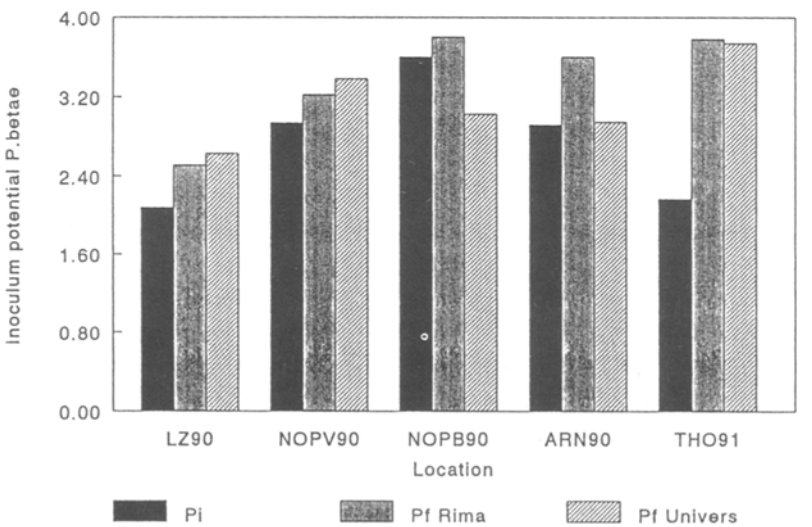

Fig. 2. The inoculum potential ($\log_{10}$ MPN + 1) of *Polymyxa betae* in soil, before (Pi) and after (Pf) growing two sugar beet cultivars at five BNYVV-infested locations. Cv Rima is partially resistant to BNYVV, cv Univers is susceptible. Locations and significances of cultivar effects are described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

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.

Populations of total and viruliferous *P. betae* hardly increased at the locations investigations in 1990, only in THO91 did populations show a considerable increase (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 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 soilborne 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 infected 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 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 that 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). The 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.

Acknowledgements

We gratefully acknowledge the critical reading of the manuscript by Prof. Dr J. C. Zadoks and Drs G. J. Bollen (Department of Phytopathology, Wageningen Agricultural University).

References

Abe H and Tamada T (1986) Association of beet necrotic yellow vein virus with isolates of *Polymyxa betae* Keskin. Annals of the Phytopathological Society of Japan 52: 235–247

Adams MJ (1991) Transmission of plant viruses by fungi. Annals of Applied Biology 118: 479–492

Adams MJ, Jones DR, O'Neill TM and Hill SA (1993) The effect of cereal break crops on barley mild mosaic virus. Annals of Applied Biology 123: 37–45

Adams MJ, Jones P and Swaby AG (1987) The effect of cultivar used as host for *Polymyxa graminis* on the multiplication and transmission of barley yellow mosaic virus (BaYMV). Annals of Applied Biology 110: 321–327

Asher MJ and Barr KJ (1990) The host range of *Polymyxa betae* and resistance in *Beta* species. Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors. Braunschweig, Germany, August 21–24 1990. Schriftenreihe der Deutschen Phytomedizinischen Gesellschaft. Band 1: 65–68

Barr DJS (1979) Morphology and host range of *Polymyxa graminis*, *Polymyxa betae* and *Ligniera pilorum* from Ontario and some other areas. Canadian Journal of Plant Pathology 1: 85–94

Bürcky K and Büttner G (1985) Ansätze zur Selektion rizomaniatoleranter Zuckerrüben während der Jugendentwicklung. I. Virustiter. Zuckerindustrie 110: 997–1000

Bürcky K and Büttner G (1988) Ansätze zur Selektion rizomaniatoleranter Zuckerrüben während der Jugendentwicklung. III. BNYVV-Gehalt in Haupt-und Seitenwurzeln. Zuckerindustrie 113: 497–500

Bürcky K and Büttner G (1991) Gehalt an Beet Necrotic Yellow Vein Virus (BNYVV) in der Hauptwurzel von Zuckerrübenpflanzen verschiedener Sorten und deren Leistung unter Rizomaniabefall im Feld. Journal of Phytopathology 131: 1–10

Campbell RN and Lin MT (1976) Morphology and thermal death point of *Olpidium brassicae*. American Journal of Botany 63: 826–832

Canova A (1966) Ricerche sulle malattie da virus delle Graminacee III. *Polymyxa graminis* Led. vettore del virus del mosaico del Frumento. Phytopathologia Mediterranea 5: 53–58

Ciafardini G and Marotta B (1988) Scanning electron microscopy of the sporosorus in *Polymyxa betae* (Plasmodiophoromycetes). Canadian Journal of Botany 66: 2518–2522

Clark MF and Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34: 421–435

Cochran WG (1950) Estimation of bacterial densities by means of 'the most probable number'. Biometrics 6: 105–116

Cooper JI and Jones AT (1983) Responses of plants to viruses: proposals for the use of terms. Phytopathology 73: 127–128

Fujisawa I and Sugimoto T (1977) Transmission of beet necretic yellow vein virus by *Polymyxa betae*. Annals of the Phytopathological Society of Japan 43: 583–586

Gibbs AJ and Gower JC (1960) The use of a multiple-transfer method in plant virus transmission studies – some statistical points arising in the analysis of results. Annals of Applied Biology 48: 75–83

Giunchedi L, De Biaggi M and Poggi Pollini C (1985) Evaluation of ELISA technique for the screening of rhizomania-tolerant sugar beet genotypes. Proceedings 48th Winter Congress IIRB, Brussels, pp. 385–390

Giunchedi L, De Biaggi M and Poggi Pollini C (1987) Correlation between tolerance and beet necrotic yellow vein virus in sugar-beet genotypes (1). Phytopathologia Mediterranea 26: 23–28

Giunchedi L and Langenberg WG (1982) Beet necretic yellow vein virus transmission by *Polymyxa betae* Keskin zoospores. Phytopathologia Mediterranea 21: 5–7

Goffart JP, Van Bol V and Maraite H (1987) Quantification du potentiel d'inoculum de *Polymyxa betae* Keskin dans les sols. Proceedings 50th Winter Congress IIRB, Brussels: 295–306

Jianping C, Swaby AG, Adams MJ and Yili R (1991) Barley mild mosaic virus inside its fungal vector, *Polymyxa graminis*. Annals of Applied Biology 118: 615–621

Keskin B (1964) *Polymyza betae* n. sp., ein Parasit in den Wurzeln von *Beta vulgaris* Tournefort, besonders während der Jugendentwicklung der Zuckerrübe. Archiv für Mikrobiologie 49: 348–374

Maury Y, Duby C, Bossenec JM and Boudazin G (1985) Group analysis using ELISA: determination of the level of transmission of soybean mosaic virus in soybean seed. Agronomie 5: 405–415

Paul H, Henken B and Alderlieste MFJ (1992) A greenhouse test for screening sugar-beet (*Beta vulgaris*) for resistance to beet necrotic yellow vein virus (BNYVV). Netherlands Journal of Plant Pathology 98: 65–75

Paul H, Van Eeuwijk FA and Heijbroek W Multiplicative models for cultivar by location interaction in esting sugar beets for resistance to beet necrotic yellow vein virus. Euphytica (in press)

Payne RW, Arnold GM and Morgan GW (1991) Genstat 5 Procedure Library Manual Release 2[2]. NAG, Oxford, 259 pp

Payne RW, Lane PW, Ainsey AE, Bicknell KE, Digby PGN, Harding SA, Leech PK, Simpson HR, Todd AD, Verrier PJ, White RP, Gower JC, Tunnicliffe Wilson G and Paterson LJ (1988) Genstat 5 Reference Manual. Clarendon Press, Oxford, 749 pp

Richard-Molard M (1987) Variétés tolérantes à la rhizomanie – Résultats d'essais, problèmes posés par leur utilisation. Proceedings 50th Winter Congress IIRB, Brussels: 111–129

Rysanek P, Stocky G, Haeberlé AM and Putz C (1992) Immunogold labelling of beet necrotic yellow vein virus particles inside its fungal vector, *Polymyxa betae* K. Agronomie 12: 651–659

Schlösser E (1990) Rizomania. XII. Effect of BNYVV and BSBV on the development of *Polymyxa betae*. Mededelingen van de Faculteit Landbouwwetenschappen van de Rijksuniversiteit Gent 55: 1069–1071

Siegel S (1956) Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York. 312 pp

Swallow WH (1985) Group testing for estimating infection rates and probabilities of disease transmission. Phytopathology 75: 882–889

Tamada T and Abe H (1989) Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus *Polymyxa betae*. Journal of General Virology 70: 3391–3398

Tamada T and Kusume T (1991) Evidence that the 75K readthrough protein in beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus *Polymyxa betae*. Journal of General Virology 72: 1497–1504

Tuitert G (1990) Assessment of the inoculum potential of *Polymyxa betae* and beet necrotic yellow vein virus (BNYVV) in soil using the most probable number method. Netherlands of Plant Pathology 96: 331–341

Tuitert G (1993) Effect of conditions during storage of infested soil on infection of bait plants by *Polymyxa betae* and beet necrotic yellow vein virus. Netherlands Journal of Plant Pathology 99: 291–301

Tuitert G and Bollen GJ (1993) 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. Netherlands Journal of Plant Pathology 99 (suppl. 3): 219–230

Tuitert G and Hofmeester Y (1992) Epidemiology of beet necrotic yellow view virus in sugar beet at different initial inoculum levels in the presence of absence of irrigation: Dynamics of inoculum. Netherlands Journal of Plant Pathology 98: 343–360

Tuitert G and Hofmeester Y (1994) Epidemiology of beet necrotic yellow vein virus in sugar beet at different initial inoculum levels in the presence of absence of irrigation: Disease incidence, yield and quality. European Journal of Plant Pathology 100: 19–53

Winner C (1988) Terminologische Fragen in der Rizomaniaforschung. Zuckerindustrie 113: 597–800