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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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