

A greenhouse test for screening sugar-beet (*Beta vulgaris*) for resistance to beet necrotic yellow vein virus (BNYVV)

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Abstract

Small differences in activity between batches of purified beet necrotic yellow vein virus (BNYVV) were observed in ELISA. A four-parameter modelled dose-response curve of purified BNYVV was used for the conversion of ELISA values to virus concentrations. Seedlings of the susceptible cultivar Regina and the partially resistant cultivars Nymphe and Rima were tested for resistance to BNYVV in a mixture of sand and infested soil. Plants were grown in a greenhouse with low nutrient supply and at temperatures below the optimum of both the vector *Polymyxa betae* and BNYVV. Root systems were small and consisted mainly of lateral roots. Significant differences in average virus concentrations were found between cultivars, both when using the complete root systems and when using either the top or the bottom part of the root systems. Average virus concentrations in 'Regina' were always significantly higher than in 'Rima' and higher than in 'Nymphe' on all occasions except one ($P < 0.05$). Differences between 'Nymphe' and 'Rima' were less evident. Variation between plants was greatest within 'Rima'. The test described in this paper can be used for the discrimination of different cultivars and for the identification of individual plants with resistance to BNYVV.

Additional keywords: rhizomania, *Polymyxa betae*, ELISA.

Introduction

Rhizomania in beet, caused by beet necrotic yellow vein virus (BNYVV) (Tamada, 1975), has become a major threat to sugar-beet (*Beta vulgaris* L.) crops in most beet growing areas of the world. The soil-borne fungus *Polymyxa betae* Keskin (*Plasmodiophoraceae*) acts as a vector of the virus. Symptoms of the disease consist of proliferation of lateral roots, constriction of the tap root and browning of the vascular system in the roots. Leaves of infested plants are light green in colour. Occasionally, when the virus becomes systemic, leaves are dark green with yellow veins. Rhizomania can cause serious losses both in root weight and sugar content. Cultural practices have limited effect on the level of infection (Müller and Gösswein, 1987) and the possibilities for controlling *P. betae* by chemicals are restricted (Schaüfele, 1987). Breeding for resistance to rhizomania is regarded as promising to control the disease (Bolz and Koch, 1983). Since 1982, such breeding activities have increased considerably and test networks for evaluating cultivars have been set up in many countries (Richard-Molard,

1987). Because sugar-beet is a cross-pollinating crop, differences in resistance to rhizomania between plants can occur within cultivars and breeding populations. In most breeding programmes for resistance, selection is usually done in field experiments with disease symptoms, root weight and sugar content as selection criteria. Greenhouse tests are also being used.

Giunchedi et al. (1985, 1987), using the enzyme-linked immunosorbent assay (ELISA) technique for virus determination in roots, reported a reduction in the virus concentration in so-called rhizomania-tolerant material. Differences in virus concentration could already be demonstrated in the seedling stage (Bürcky and Büttner, 1985). These results have stimulated the development and use of greenhouse screening tests, in which virus concentration in the roots is used as a selection parameter for resistance to rhizomania. ELISA readings were interpreted quantitatively either by converting the readings to absolute virus concentrations by means of a dose-response curve of purified virus, applying linear regression (Giunchedi et al., 1985, 1987), or by classifying the readings according to the response of different dilutions of purified virus and expressed as relative virus concentrations (Bürcky and Büttner, 1988). Both tap roots and lateral roots could be used in preparing samples for virus assays. Virus concentrations were lower in tap roots than in lateral roots (Büttner and Bürcky, 1990; Giunchedi et al., 1985, 1987). Bürcky and Büttner (1988) recommended the use of lateral roots in experiments in which individual plants are selected for resistance, because sometimes in tap roots no virus could be detected. However, the difference in virus concentration of the lateral roots between a resistant and a susceptible cultivar decreased with time, which would mean that for the discrimination between a resistant and a susceptible cultivar, lateral roots could only be used during a limited testing period. Furthermore, the preparation of samples from lateral roots was more laborious than from tap roots (Bürcky and Büttner, 1985, 1988).

The aim of the present study was to develop a quantitative greenhouse screening test in which individual plants could be tested for resistance to rhizomania and in which lateral roots would be used for sampling. Different batches of purified virus were tested for their use in dose-response curves and special attention was given to the modelling and statistical analysis of ELISA data. Following the approach of Toxopeus and Lubberts (1979), who described a method for testing resistance to the beet cyst nematode *Heterodera schachtii* Schm. in seedlings, test plants were grown in sand with low nutrient supply, to obtain root systems consisting mostly of lateral roots. By growing the plants at temperatures below the optimum of both vector-fungus and virus it was aimed to get only a moderate development of the disease, so that the period in which resistant and susceptible cultivars could be discriminated, would not be restricted (Bürcky and Büttner, 1985). Furthermore, it was investigated whether subsamples of the roots could be used instead of the whole root system, which would facilitate the use of lateral roots.

Materials and methods

Purified virus. Three batches of purified BNYVV, named 90-1, 90-2 and 90-3, were received from Dr D. Peters (Department of Virology, Wageningen Agricultural University). Virus concentrations of the batches were spectrophotometrically estimated at 2.0, 0.8 and 1.0 mg ml⁻¹ (E260, 0.1% = 2.8). Batches were stored at 4 °C.

Dilutions of 1600, 800, 400, 200, 100, 50, 25, 5 and 1 ng ml⁻¹ were made in phosphate buffered saline (plus 0.05% Tween 20) (PBS-Tween), and tested with ELISA in three replicates.

Plant material and cultivation of test plants. Seeds of the sugar-beet cultivars Regina (Hilleshög-Sweden, susceptible), Nymphe (Hilleshög-Sweden, partially resistant) and Rima (SES-Belgium, partially resistant) were sown in heat-sterilized (overnight, 105 °C) coarse sand. Seeds of 'Regina' and 'Nymphe' were disinfected with Dithane M45 (Duphar). For 'Rima' coated seeds were used. Soil was collected from a field in Nagele (Noord-Oost Polder), earlier described by Beemster and De Heij (1987) as containing high levels of *P. betae* infested with BNYVV. The soil was air-dried, mechanically ground and mixed thoroughly with heat-sterilized coarse sand in a ratio of 1 : 9 (v/v). Seedlings were transplanted to black PVC containers (40×40×150 mm, Kelder Plastibox) with folded bottom, filled with the soil-sand mixture. For the production of healthy plant material, seedlings were transplanted to containers with sand only. One seedling was planted per container. Each container was placed in an aluminium tray (80×80×29 mm, Ekco) (Fig. 1A). Twice a week the trays were supplemented to half height with a 0.1 dilution of a Steiner nutrient solution (pH 7). Tests were carried out in a climate-controlled greenhouse. Temperatures were 22 °C at day time (10 h) and 17 °C at night (14 h), which is below the optimum temperature of both the vector and the virus (Asher and Blunt, 1987; Horak, 1980). Natural daylength was extended to 16 h, with a minimum light intensity of 10 Watt m⁻².

Experimental design of greenhouse experiments. In a first experiment, 120 plants of each of the cultivars Regina, Nymphe and Rima were tested in a completely randomized design. After 16, 22, 28, 34 and 40 days, 24 plants of each cultivar were sampled at random. The complete root systems of the plants were used. In a second experiment, 60 plants of each of the three cultivars were tested in a completely randomized design. After 22 and 29 days, 30 plants of each cultivar were sampled at random. Some 100 mg from the upper part of the root system and the remaining part were sampled separately. In each experiment, samples of all sampling times were stored and analysed simultaneously in ELISA at the end of the experiment. Non-infested plants were kept separately from the main experiments.

Root sampling. The containers were placed in a tray filled with water. Then, the soil and sand were washed away from the roots under a tap (Fig. 1B). The roots of each plant were checked for the presence of cystosori of *P. betae* by direct observation under an inverted microscope (Zeiss ID02). Roots were cut from the plants, dried carefully with tissue paper, weighted and crushed in Potter tubes with PBS-Tween in a ratio of 1 : 20 (w/v). Stock solutions of healthy plant sap were obtained by squeezing roots of non-infested plants in a garlic press. Samples and stock solutions were stored at -18 °C in Eppendorf tubes.

Virus assays. ELISA (Clark and Adams, 1977) was performed as described by Alderlieste and Van Eeuwijk (in press). Coating and conjugated antibodies (Sanofi commercial antiserum) were diluted 130 and 1000 times, respectively. Para-nitrophenyl-phosphate (Sigma, 1 mg ml⁻¹) was used as substrate. Incubation times

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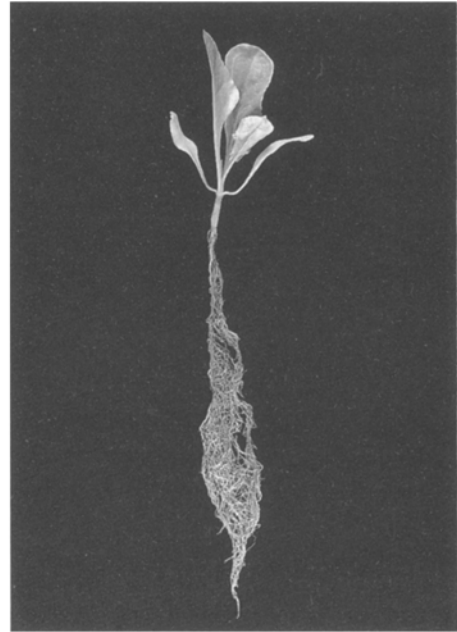


Fig. 1A/B. A: Sugar-beet seedling of 'Regina' in a container filled with a mixture of sand and infested soil, placed in a tray with nutrient solution (27 days after transplanting) (picture on the left); B: Idem, with roots washed free of sand and soil (picture on the right).

and temperatures were according to Büttner and Bürcky (1987). Only the inner 60 wells of the microtiter plates (Costar 3590) were used. Outer wells were filled with PBS-Tween in all steps of ELISA, except for the last one, where these wells were filled with substrate.

Samples and stock solutions were thawed and centrifuged (2 minutes, Micro-spin 24, Sorvall Instruments). In the assays of the root samples, a dilution series of 1600, 800, 400, 200, 100, 50, 5 and 0 ng ml⁻¹ of virus batch 90-2 was included as a standard on each microtiter plate. Since plant sap components influence ELISA (Alderlieste and Van Eeuwijk, in press; Büttner and Bürcky, 1987; Koenig et al., 1984), virus standards were made up in a 0.05 (v/v) solution of healthy plant sap of 'Regina', diluted in PBS-Tween, to obtain a dilution comparable to that of the root samples. In the assays, dilutions of purified virus and the virus standards and samples of the first greenhouse experiment were tested in duplicate. Virus standards and samples of the second greenhouse experiment were tested without duplication. All dilutions, standards and samples were randomized on the plates, with duplicates in adjacent wells. Reactions were stopped with 0.05 ml 5M NaOH per well, when the 800 ng ml⁻¹ standard of batch 90-2 had reached a value of approximately 1.0. Readings were performed with an Anthos 2001 multiscan photospectrometer at 405 nm, with 620 nm as reference, in combination with the software package ELISA+ (Meddata Inc.), installed on a microcomputer.

Data analysis. Data were transferred to a main-frame computer (VAX, Digital). Readings of the front wells (B1-G1) were used for blanking the rows. When duplicates were used, the means of the two readings were used in the analysis. As is extensively described by Alderlieste and Van Eeuwijk (in press), a four-parameter logistic model (Dudley et al., 1985), which is characterised by the lower asymptote, the inflexion point, the slope parameter and the upper asymptote, fits well to the dose-response curve of purified BNYVV. The same model was used for the conversion of ELISA-readings to concentrations, using the virus standards for modelling the dose-response curve. No virus, 0 ng ml^{-1} , was read as 1 ng ml^{-1} . Readings equal to or below the estimated lower limit of the curve were replaced by that lower limit + 0.001. Curve-fittings and analyses of variance were carried out with the statistical package GENSTAT (GENSTAT 5, Rothamsted Experimental Station, U.K.). Pairwise comparisons between cultivars on the different sampling times were made with least significant differences (LSD).

Results

Purified virus. Figure 2 shows the response in ELISA of dilutions of the three batches of purified beet necrotic yellow vein virus. Differences in response between the batches were observed. Slopes of the curves were similar. Before the means were used in analyses, the correlation between duplicate readings were computed. Correlations were higher than 0.99. After application of the four-parameter logistic model to the

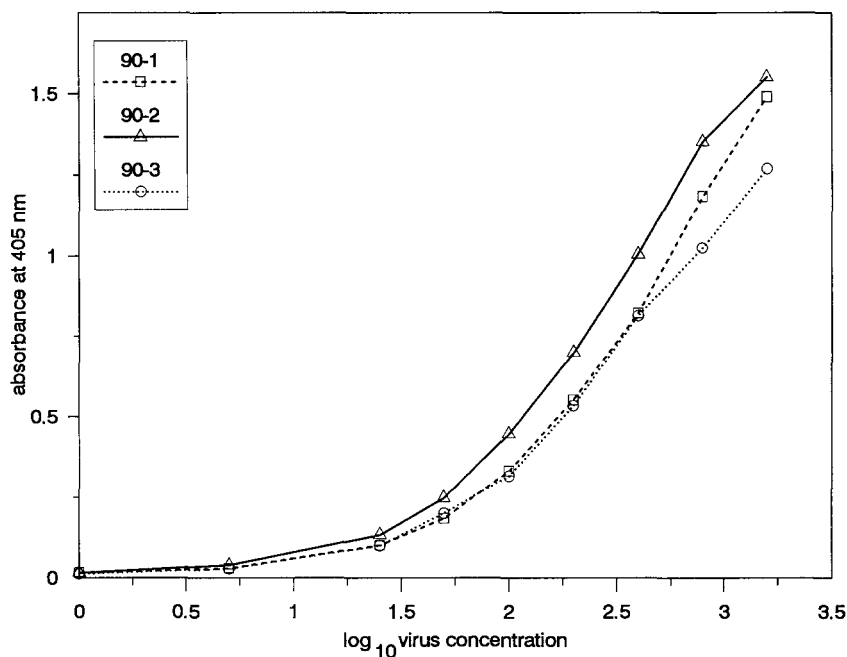


Fig. 2. Dose-response curves of three batches of purified BNYVV (means of three replicates, original data on virus concentration in ng ml^{-1}).

data presented in Fig. 2, curve fittings were 100.0, 99.9, and 99.8% for the batches 90-1, 90-2 and 90-3, respectively.

Greenhouse experiments

Observations on the roots. All three cultivars, Regina, Nymphe and Rima, were susceptible to *P. betae*. Cystosori, the resting spores of the fungus, were detected in the roots of all plants at all sampling times. Up till 22 days in both experiments many of the cystosori were still immature. Most cystosori were found in the first lateral roots, branching off the tap root.

Root weight increased with time (Table 1). Mainly lateral roots were formed, and the tap roots only slightly thickened during the experiments. In the first experiment, average root weight of 'Nymphe' was significantly higher than that of 'Regina' and 'Rima' at the first sampling time and higher than the average root weight of 'Regina' at the fourth sampling time ($P < 0.05$). In the second experiment, average root weights of 'Nymphe' and 'Rima' were significantly higher than that of 'Regina' at both sampling times ($P < 0.05$). For root weight, no significant interaction between sampling times and cultivars was found in either experiment. Increased browning of the roots was observed in the course of the experiments, but no roots died.

Results of virus assays. Correlations between duplicate readings in the first experiment were higher than 0.99. In both experiments the dose-response curves of purified virus were adequately described by the four-parameter logistic model. Average curve fittings were 99.8% in both experiments. Standard concentrations of 0 ng ml⁻¹ were estimated from 0 to 2 ng ml⁻¹. Samples that gave experimental responses below 4 ng ml⁻¹ were considered to be free of virus. All virus concentrations of the plant samples were within the range of the dose-response curves. For statistical analyses, the log₁₀ of the concentrations was taken. Zero values were read as 1 ng ml⁻¹.

Figure 3 illustrates the development of BNYVV-concentrations with time in the different cultivars, tested in the first experiment. Average virus concentrations increased with time for all three cultivars from day 16 to day 34. Virus concentrations were similar at the last two sampling times. Virus was found in all samples of 'Regina',

Table 1. Average fresh root weight (mg) of sugar-beet seedlings, with 95%-confidence intervals, at different sampling times during tests for resistance to BNYVV.

Cultivar	Experiment 1					Experiment 2	
	Days after transplanting					Days after transplanting	
	16	22	28	34	40	22	29
Regina	129 ± 10	258 ± 26	293 ± 32	338 ± 36	430 ± 56	304 ± 26	508 ± 41
Nymphe	145 ± 13	258 ± 22	328 ± 33	407 ± 37	487 ± 45	343 ± 23	573 ± 37
Rima	126 ± 11	263 ± 23	330 ± 28	389 ± 39	451 ± 43	347 ± 26	563 ± 35

Experiment 1: 24 plants per cv. per sampling time; Experiment 2: 30 plants per cv. after 22 days, and 28, 29 and 30 plants for cv. Regina, Nymphe and Rima, respectively, after 29 days.

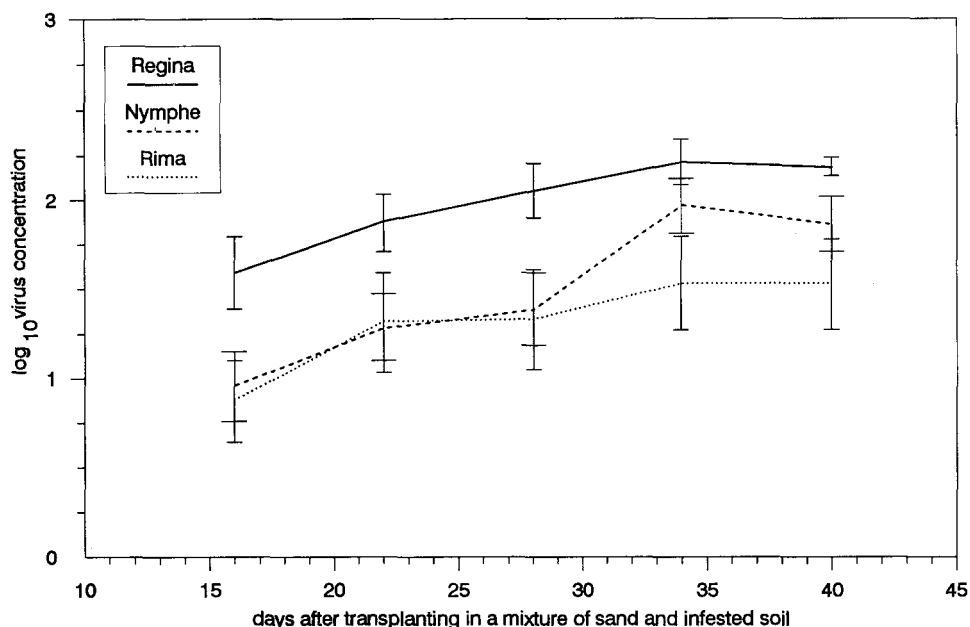


Fig. 3. Average virus concentrations in the root systems of sugar-beet seedlings at different sampling times, with 95%-confidence intervals (original data on virus concentration in ng ml^{-1}).

but especially at early sampling times, not in all samples of 'Nymphe' and 'Rima'. Only at the fourth and fifth sampling time the virus could be detected in all plants. Average virus concentrations in 'Regina' were significantly higher than in 'Nymphe' and 'Rima' at all sampling times, except on day 34, when only a significant difference with 'Rima' was found. On days 34 and 40, 'Nymphe' and 'Rima' differed significantly ($P < 0.05$) in virus concentration. No significant interaction between the sampling times and cultivars could be detected. Variation, as expressed by the width of the confidence interval, was higher in 'Rima' than in the other cultivars.

Results of the virus assays on the samples of the second greenhouse experiment are presented in Figs 4a and 4b. Histograms were used to depict the variation in virus concentration between plants within cultivars. Increases in virus concentration between the sampling times were observed for all cultivars and in both parts of the root system. As in the first experiment, all samples of 'Regina' contained virus, but no virus could be detected in several samples of 'Nymphe' and 'Rima'. Average virus concentrations in 'Regina' were significantly higher than in 'Nymphe' and 'Rima', at both sampling times and in both parts of the root system. Although average virus concentrations in 'Nymphe' were higher than in 'Rima' in both parts of the roots, significant differences were only found in the lower part of the roots, at both sampling times. No interaction was found between sampling times and cultivars or between sample types and cultivars. As in the first experiment, the highest variation was found in 'Rima'. Distribution of the data of 'Rima' was less regular than for 'Regina' and 'Nymphe', and the data of the lower part of the root system even suggested a segregation between resistant and susceptible plants.

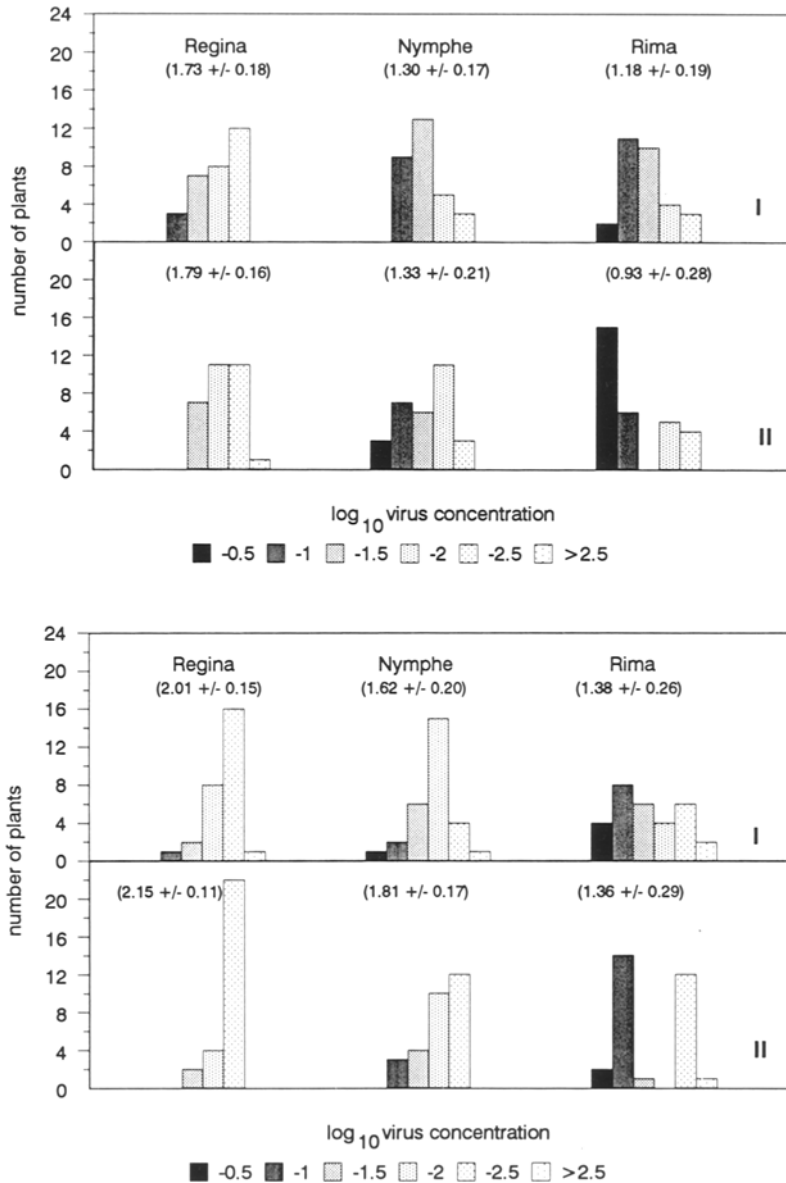


Fig. 4A/B. A: Average virus concentrations (original data on virus concentration in ng ml^{-1}), with 95%-confidence intervals, and frequency distribution of concentrations of BNYVV, in 100 mg of the upper part (I) and in the remaining part (II) of the root systems of sugar-beet seedlings on day 22 ($n = 30$ plants per cultivar); B: Idem on day 29 ($n = 28, 29$ and 30 plants for 'Regina', 'Nymphe' and 'Rima', respectively).

Discussion

Differences in response were detected between batches of purified virus. Estimates of virus concentrations of the batches were made with the help of a spectrophotometer, while comparison of activities were made in ELISA. These two methods could respond differently, which might be the result of damage of virus particles (Korpraditskul et al., 1980) or to aggregating of virus particles. When used as standard in dose-response curves, the use of different batches will result in different outcomes of estimated virus concentrations in plant samples. However, the differences between batches were small, so that the error due to the use of different batches is not considered to be a problem in quantitative assays of beet necrotic yellow vein virus. Another error in the virus assays might be a decline in activity of the virus in the batches during storage. No decline in activity of batch 90-2, estimated by the response of different dilutions, was observed within the time span of half a year in which the experiments in this study were carried out. The storage of samples therefore is thought to be of little influence on the outcome of the virus assays.

The four-parameter model proved to be appropriate for estimating virus concentrations. High percentages of curve fitting were obtained in all virus assays. In modelling dose-response curves the four-parameter model should be preferred to the linear regression model (Giunchedi et al.; 1985, 1987), because of the non-linear relationship between absorbance values and virus concentrations. The use of the four-parameter model could also be used for relative virus concentrations (Bürcky and Büttner, 1988). High correlations were found between duplicate samples. Therefore, single samples seem to be sufficient, although an internal check within the assay is no longer possible then.

The method of Toxopeus and Lubberts (1979), growing seedlings under low nutrient supply, was successful in obtaining root systems with mainly lateral roots. Significant differences in average root weights between cultivars were observed. However, no significant interactions were found between sampling times and cultivars, indicating that the growth rate of the cultivars did not differ, despite the differences in virus concentration. The roots in the second experiment were bigger than those in the first experiment. This could be explained by differences in nutrient supply. During the second experiment the relative humidity in the greenhouse was lower than in the first experiment and more nutrient solution was used to fill the trays. Virus concentrations were similar in both experiments.

Due to temperatures below the optimum of both the fungus and the virus, only moderate levels of virus were obtained. Concentrations of all plant samples were within the range of the dose-response curves. The virus could be detected in all plants of the susceptible cultivar Regina, but especially at early sampling times, not in all plants of 'Nymphe' and 'Rima'. Differences in virus concentrations between the susceptible cultivar Regina and the partially resistant cultivars Nymhe and Rima were significant at all times, except the fourth sampling time in the first experiment. Differences between 'Nymphe' and 'Rima' were less evident. The absence of interaction between sampling time and cultivar indicated that the choice of sampling time is not critical for the present greenhouse test. In view of the first experiment, however, it is suggested that sampling should not take place early after transplanting. At early sampling times, virus concentrations might be too low for discrimination between

partially resistant cultivars or between susceptible and resistant plants, and the same problem might occur as with the use of tap roots (Bürcky and Büttner, 1988).

The second experiment showed that a subsample of lateral roots could be used instead of the whole root system. No interaction between the sampling times and the two sample types were observed and 'Regina' could be distinguished from the other cultivars with either sample type. Because a better discrimination between 'Nymphe' and 'Rima' was found with the lower part of the roots, it is suggested to take a subsample of lateral roots in the lower part of the root system. This would also facilitate the maintenance of selected plants. It was not possible to differentiate 'Rima' and 'Nymphe' under all circumstances, using average virus concentration as a criterium. Variation, as expressed by the width of the confidence interval, was always higher in 'Rima' than in 'Nymphe'. The two peaks in the distribution of the data of 'Rima' suggested the presence of both resistant and susceptible plants in the same population. It is suggested, that in evaluation tests, next to the average virus concentrations of the cultivars, also the variation in virus concentration between plants within a cultivar should be taken into account. Low virus concentration may be caused either by partial resistance to the virus or to the vector *P. betae*. Since cystosori of the fungus could be detected in the roots of all cultivars, it is assumed that resistance to rhizomania in 'Nymphe' and 'Rima' is based on resistance to the virus.

The present method, in which small root systems, consisting mainly of lateral roots, are used for sampling, adequately discriminates susceptible and resistant cultivars and can be used for the selection of individual plants. With the chosen culturing conditions, the period in which different cultivars could be discriminated, did not seem to be very much restricted, and a subsample of the roots could very well be used instead of the complete root system.

Virus concentrations will not only be influenced by cultural conditions, but also by the level of inoculum. Although little is known about the amount of spores present in the soil, the present method seemed to provide sufficient inoculum to obtain infection in all plants tested.

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