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Breaking of *Beet necrotic yellow vein virus* resistance in sugar beet is independent of virus and vector inoculum densities

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Abstract *Beet necrotic yellow vein virus* (BNYVV) is transmitted by *Polymyxa betae* to sugar beet, causing rhizomania disease. Resistance-breaking strains of BNYVV, overcoming single (*Rz1*) or double (e.g. *Rz1*+*Rz2*) major resistance genes in sugar beet have been observed in France and recently in the USA and Spain. To demonstrate if resistance-breaking is dependent on inoculum density, the inoculum concentration of BNYVV and *P. betae* in soil samples where resistance-breaking had been observed was estimated using the most probable number (MPN) method. The MPN-values obtained displayed highly significant differences with respect to the virus concentration in various soils and did not correlate with the ability to overcome resistance. Virus quantification in susceptible plants demonstrated that soils containing resistance-breaking isolates of BNYVV did not produce higher virus concentrations. The MPN assay was repeated with *Rz1*+*Rz2* partially-resistant sugar beets to see if the resistance-breaking is concentration-dependent. There was no correlation between soil dilution and increased virus concentration in *Rz1*+*Rz2* plants produced by BNYVV resistance-breaking strains. Determination of the absolute *P. betae* concentration by ELISA demonstrated that all resistance-breaking soil samples contained elevated concentrations. However, the calculation of the proportion of viruliferous *P. betae* did not show a positive correlation with the resistance-breaking ability. Finally resistance-breaking was studied with susceptible, *Rz1 and Rz1*+Rz2 genotypes and standardised rhizomania inoculum added to sterilised soil. Results from these experiments supported the conclusion that resistance-breaking did not correlate with virus concentration or level of viruliferous *P. betae* in the soil.

Keywords *Beta vulgaris* ssp. *vulgaris* · Most probable number · *Polymyxa betae* · *Rz1*

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Introduction

Economically, rhizomania is one of the most important sugar beet diseases world-wide. In 1958 *Beet necrotic yellow vein virus* (BNYVV) was identified in Italy as the causal agent for severe yield and sucrose reductions in sugar beet, commonly known as rhizomania. The disease has spread to all important sugar beet-growing areas in Asia, North America and Europe (Asher et al. 2003; Lennefors et al. 2000; Nielsen et al. 2001 and Tamada 1999). BNYVV is transmitted by the obligate biotrophic plasmodiophorid *Polymyxa betae* Keskin (Keskin 1964; Tamada 1975). Yield reduction and rhizomania symptoms in susceptible cultivars are

dependent on the presence and density of viruliferous *P. betae* (Tuitert 1990). The vector itself hardly affects sugar beet growth (Rush 2003). Along with *P. betae* and hence BNYVV density in soil, factors such as soil moisture and soil temperature also play an important role regarding rhizomania symptom severity (Tuitert 1990).

Rhizomania is controlled by growing sugar beet cultivars which contain partial quantitative resistance. The major dominant resistance gene *Rz1*, is the main BNYVV resistance gene in commercial cultivars (Lewellen et al. 1987; Scholten et al. 1996). Additionally, wild beet accessions such as *Beta vulgaris* ssp. *maritima* WB41 and WB42 serve as sources for additional resistance genes *Rz2* and *Rz3*, respectively (Lewellen et al. 1987; Scholten and Lange 2000; Gidner et al. 2005). Thus, marketable rhizomania-resistant sugar beet varieties contain *Rz1* or occasionally a combination of two resistance genes (*Rz1*+*Rz2*). Nevertheless, all known major resistant genes against the virus, both on their own or in combination, provide only partial resistance and are unable to prevent BNYVV infection entirely. Usually the cultivars possess the ability to reduce the virus replication in secondary rootlets and inhibit virus spread to the taproot (Heijbroek et al. 1999; Scholten et al. 1994). Thus it is generally assumed that the inoculum potential in soils increases continuously.

Molecular analysis of the BNYVV genome has revealed three major pathotypes: BNYVV-A, -B, and —P. The sequence variation within pathotypes A and P is greater than within different B-type isolates (Kruse et al. 1994; Koenig and Lennefors 2000; Schirmer et al. 2005). The A-type occurs in southern Europe, the Benelux countries, Asia and the USA, whereas the BNYVV B-type is found in Germany, the upper Rhine valley in France and Switzerland. Koenig et al. (1997) initially described the European BNYVV P-type, which occurs only in a small region near Pithiviers in France and recently at four sites in the UK (Ward et al. 2007). In Europe, BNYVV-P is the only pathotype that contains an additional RNA 5 (Koenig et al. 1986). BNYVV-P shows an enhanced rate of spread in plants due to the additional RNA 5 encoding a second pathogenicity factor (Heijbroek et al. 1999; Tamada et al. 1996).

In addition to reports about increased aggressiveness of French BNYVV P-type, BNYVV A-types in the USA (Imperial Valley, California and Minnesota), able to overcome *Rz1* in field and inducing elevated BNYVV levels in secondary rootlets in greenhouse resistance tests have recently been identified (Liu et al. 2005; Liu and Lewellen 2007). Previously, we have compared resistance-breaking abilities of soil samples containing A-type isolates from all major sugar beet-growing areas, where resistance-breaking has been observed (France: Pithiviers, US: Imperial Valley and Minnesota) plus an additional soil sample from Spain (Daimiel) (Julian Ayala, personal communication) in sugar beet cultivars carrying *Rz1* and *Rz1*+*Rz2* BNYVV resistance under controlled greenhouse conditions (Pferdmenges et al. 2008). We demonstrated comparable resistance-breaking abilities of US and Spanish soil samples containing aggressive BNYVV A-type isolates, as well as in soil containing a P-type isolate in an *Rz1* cultivar, through elevated virus concentrations in rootlets and reduced taproot growth. However, it is still unclear how the resistance-breaking abilities in these soils arise. There is a growing body of evidence that genetic changes in the RNA 3 encoded viral pathogenicity factor P25 are associated with BNYVV ability to overcome plant resistance (Acosta-Leal and Rush 2007; Chiba et al. 2008), though final experimental proof applying field isolates is still lacking. On the other hand, although using an artificial experimental system with zoospore cultures and hydroponics, Scholten et al. (1994) found evidence for the involvement of enhanced vector concentration and/or secondary multiplication, and that possible increased BNYVV transmission was involved in the resistance-breaking phenomenon.

To address the concentration of viruliferous *P. betae* in soil-samples, few methods are available. Either bait plant bioassays using soil dilutions are applied to estimate the most probable numbers (MPN) of infective propagules (Tuitert 1990) or polymerase chain reaction (PCR) is used to estimate *P. betae* concentration in plant material and soil (Mutasa et al. 1995; Mutasa-Gottgens et al. 2000; Kingsnorth et al. 2003). In a soil sample from The Netherlands, Tuitert (1990) estimated a proportion of 10–15% of the root-infecting *P. betae* population to be viruliferous. Experiments to determine and compare the BNYVV and *P. betae* inoculum concentration in soils displaying resistance-breaking abilities are still lacking. In addition, no resistance tests applying soils containing resistance-breaking BNYVV strains in varying con-

centrations have been carried out to date to prove the hypothesis that increased pathogenicity is related to high inoculum density. All previous resistance tests have been carried out in natural field-derived soil; thus an additional synergism of superinfections with other soil-borne pathogens such as *Rhizoctonia solani*, *Fusarium* spp. and nematodes could not be entirely excluded.

The aim of this study was to detect a possible relationship between the severe rhizomania symptoms observed in BNYVV-resistant sugar beet cultivars in resistance-breaking soils from France, Spain and the USA in the field with the ability to induce elevated BNYVV concentrations, and taproot weight reductions in greenhouse resistance tests. This was done by examining BNYVV inoculum concentration in soil and *P. betae* reproduction abilities in lateral roots and the percentage of viruliferous *P. betae*. The results were compared to reference rhizomania soils from Germany and Italy which do not display increased aggressiveness towards cultivars containing *Rz1* and *Rz2*. The MPN assay was repeated with *Rz1+Rz2* plants to test the hypothesis: if overcoming resistance solely depends on BNYVV concentration, the occurrence of plants with significant BNYVV concentrations ceases at a dilution step well before the out-dilution point observed using the MPN method with susceptible plants. Subsequently the virus concentration in collected lateral roots was standardised and served as the inoculum for the infestation of sterile soil. A time-series harvest was conducted within this test under standardised inoculum densities to observe the temporal virus propagation and systemic spread in sugar beet.

Materials and methods

MPN method - sample preparation and serial dilutions

To estimate the number of infective BNYVV units and *P. betae* zoospores in soils from different origins, the MPN method as described by Tuitert (1990) was used in a modified form. The ratio for the serial soil dilution was reduced from 10 to 5 and the number of dilution steps was raised from 5 to 6 to increase the coverage of different inoculum densities. For each dilution step 10 plants were planted into single pots so that each plant served as an independent replicate in a fully randomised experimental design. Soil samples were air-dried and sieved over 2 mm sieves. Autoclaved sand (coarse size 1-2 mm) served as the control. Each soil sample was thoroughly mixed with dried sterile sand in 40 l plastic bags, starting from the highest (5^{-1}) to the lowest concentration (5^{-6}). Each test plant was grown in 300 g of mixed soil from each dilution in plastic boxes (size 4 cm × 4 cm × 16 cm). The MPN calculation was conducted using an MPN calculator, based on the maximum likelihood equation following the MPN calculations of Hurley and Roscoe (1983). This equation was applied for the development of the MPN calculator (MPN Calculator version VB6) which was used to generate the data of this study (http://www.i2workout.com/mcuriale/mpn/index.html).

Soil origin

Soil samples recently collected from sugar beet-growing areas in Italy (Rovigo, referred to as R), Germany (Groß-Gerau; GG), Spain (Daimiel; D), USA (Imperial Valley: IV and Minnesota: MN) and France (Pithiviers; P) were chosen for serial dilution experiments. The soil samples used were the same as those used in resistance-breaking studies (Pferdmenges et al. 2008).

Plant material

To analyse the concentration of infectious BNYVV and *P. betae* units in soil a BNYVV-susceptible sugar beet line (*rz1rz1*) and a BNYVV double-resistant variety *Rz1rz1+Rz2rz2* (referred to as *Rz1+Rz2*) were used. In experiments using an adjusted BNYVV inoculum, a third single-resistant variety *Rz1rz1* (referred to as *Rz1)* was included. The cultivars in this study were those used for cross-classified resistance tests (Pferdmenges et al. 2008) and also by Liu et al. (2005) and Liu and Lewellen (2007) for characterisation of BNYVV-increased pathogenicity in IV and MN soil samples.

Bioassay

Seven day-old seedlings, which had been germinated in sterile silica sand, were transplanted into fully water-saturated soil. At 7 days after planting, sugar beets were sprayed with fungicides (0.2 gl^{-1} Tachigaren 70 W.P., Sumitumo, Düsseldorf, Germany) to prevent *Aphanomyces* spp. and *Pythium* spp. infections. Plants

were grown in a climate chamber at day and night temperatures of 24°C and 20°C, respectively, and a 16 h photoperiod with supplementary light.

Quantification of BNYVV and *Polymyxa* betae zoospores

The quantitative determination of BNYVV concentration in sugar beet secondary rootlets via DAS-ELISA was conducted as described (Pferdmenges et al. 2008). After 4 weeks of greenhouse cultivation, sugar beet plants were harvested individually. Sand and soil were thoroughly removed by washing with running tap water and leaves and hypocotyls discarded. Lateral roots were separated from the taproot and dried with paper towels. The quantification of *P. betae* by TAS-ELISA via specific detection of fungal glutathione-S-transferase (GST) was carried out as described by Pferdmenges et al. (2008).

Resistance tests with standardised inoculum

Lateral sugar beet roots from susceptible sugar beet cultivars, previously cultivated for 12 weeks in R, GG, D, IV, MN and P soil samples and in sterile sand, were used to standardise the BNYVV inoculum. To obtain the inoculum, beets were harvested, washed with tap water and leaves discarded. Lateral roots were separated from the taproot, collected and air-dried at room temperature for 3 days. Dried lateral roots were cut into small pieces (max. 2 mm in length) and thoroughly mixed. An aliquot was used for determining the BNYVV concentration by quantitative DAS-ELISA. Each homogenised lateral root bulk was then diluted in moist soil consisting of sand: clay mixture (1:2) to a final concentration of 70 ng BNYVV kg^{-1} sterilised damped soil. Plants to be harvested after 4 and 8 weeks were potted into 1 kg of soil and plants to be harvested after 12 weeks were potted into 2.5 kg of soil per pot. Homogeneous dispersal of the inoculum was achieved by pouring an equal mixture of dried root samples and 150 ml tap water onto the top soil layer in each pot; 10 sugar beet seedlings were planted into each pot. For each variant (root origin×harvest date), 10 individual sugar beet plants served as replicates at harvest.

The bioassay was conducted as described above. After 4, 8 and 12 weeks of greenhouse cultivation, sugar beet plants were harvested individually and the BNYVV concentration in the lateral roots determined by quantitative DAS-ELISA as described above. The time-series harvest was carried out to follow changes in virus concentration over time in lateral roots and to obtain taproots for weight determination and subsequent Tissue Print Immuno Assay (TPIA) in order to detect virus effects on root growth and systemic virus spread, respectively, indicative overcoming resistance.

Tissue Print Immunoassay (TPIA)

The systemic spread of BNYVV within sugar beet taproots was analysed using TPIA. Longitudinal sections of 12 week-old sugar beet taproots from the resistance test with standardised inoculum were firmly pressed onto positively charged nylon membranes (Hybond N+, Roche). TPIA was carried out exactly as described by Kaufmann et al. (1992). BNYVV-specific antibodies were detected with a chromogenic substrate reaction, leading to a bluish colour development on the blot surface if virus particles were present.

Data analysis

The data were analysed using SAS 10.0 (SAS Systems, Cary, NC). The PROC GLM and an unvaried procedure (PROC UNIVARIATE) were conducted to test for normality. A boxcox-macro transformation (Piepho and Ogutu 2003) was followed until all data displayed a normal distribution. The ANOVA was conducted with transformed data using the PROC MIXED procedure. All statistically analysed data in this study are presented in the reverted transformed format.

Results

BNYVV inoculum density in resistance-breaking soils and virus concentration in susceptible plants

In order to detect a possible relationship between the increased BNYVV pathogenicity derived from different soils with the inoculum density, all soil samples (GG, R, D, IV, MN and P) were used to perform an MPN assay with a susceptible sugar beet line in two independent experiments. After 4 weeks in the growth

chambers, sugar beet plants displayed obvious differences in phenotype and strength of BNYVV symptoms, depending on soil origin. Due to severe infections with BNYVV or other soil-borne pathogens and resulting plant death, the number of test plants in some soil versus dilution variants was reduced to nine (Table 1). For MPN calculations, the number of infected plants necessary in each treatment was determined. In order to detect correlations between inoculum concentration and symptom severity observed in the different soil dilution steps, and correlations to 12 week greenhouse resistance tests carried out previously (Pferdmenges et al., 2008), the virus concentration was additionally assayed quantitatively. With some of the high soil dilutions, not all plants were found to be infected with BNYVV: these non-infected sugar beet plants had to be excluded from the evaluation of all soil-dilution variants to estimate the average BNYVV-concentration per variant. The calculated MPN-values shown in Table 1 indicated large variations in the BNYVV concentration in soil samples derived from different geographic origins. The German GG-soil sample displayed the lowest inoculum concentration of all soils tested, estimated at 11 infective units (iu) g^{-1} soil. Remarkably, the D-soil sample contained a BNYVV concentration that was 520 orders of magnitude greater than that of the GG-soil sample.

By using a rhizomania-susceptible sugar beet line, a high variation in virus concentration among almost all soils was shown which did not correlate to the serial dilution of the soil (Fig. 1). If the BNYVV concentrations induced by the different soils in the lowest dilution ($D1=5^{-1}$) were compared, P-type soil produced the highest value. In higher dilutions (5^{-1} until 5^{-3}), however, the virus concentrations induced by other soil samples (except for R and P) slightly increased. The BNYVV concentration then declined until the virus was diluted from the soil, except for soil D. Interestingly, soils containing resistance-breaking isolates of BNYVV did not produce higher concentrations of virus in the lateral roots of the susceptible lines.

BNYVV inoculum densities in resistance-breaking soils and virus concentrations in *Rz1+Rz2* plants

To investigate whether BNYVV isolates from soils D, P, IV and MN were able to overcome resistance and produce elevated BNYVV concentrations not only after 12 weeks greenhouse resistance tests (Pferdmenges et al. 2008) but also in serial soil dilutions, a variation of the 4-week MPN-assay was carried out with an *Rz1+Rz2* cultivar (Table 2 and Fig. 2). *Rz1+Rz2* plants had much higher concentrations of BNYVV after 4 weeks in the growth chamber than at 12 weeks in the greenhouse. However, the virus concentrations in lateral roots in the different soil dilutions did not reflect a positive correlation between virus concentration in soil and the ability to produce detectable

Table 1 BNYVV concentration in soil samples from different geographic origins, determined with a susceptible sugar beet genotype (*rz1rz1*) and DAS-ELISA measurement after 4 weeks greenhouse cultivation to calculate the MPN-values by means of number of infected plants / number of harvested plants shown for each soil variant in different dilution steps

Soil origin						
Dilution	R	GG	D	IV	MN	P
5^{-1}	10*/10**	9/10	10/10	10/10	9/9	10/10
5^{-2}	9/10	3/10	10/10	10/10	10/10	10/10
5^{-3}	6/10	1/10	10/10	9/10	4/10	10/10
5^{-4}	5/10	0/10	10/10	7/10	3/10	2/10
5^{-5}	0/10	0/10	9/10	3/10	0/10	2/9
5^{-6}	0/10	0/10	1/9	0/10	0/10	0/10
MPN g^{-1}soil	110 a***	11 b	5200 c	510 d	98 a	340 d

Dilution ratio = 5

*Number of infected sugar beets

**Number of single-harvested sugar beets

***Means with the same letter of Most Probable Number (MPN g^{-1} soil) are not significantly different (LSD 0.05); numbers in bold represent lowest soil dilution step, where out-dilution was observed

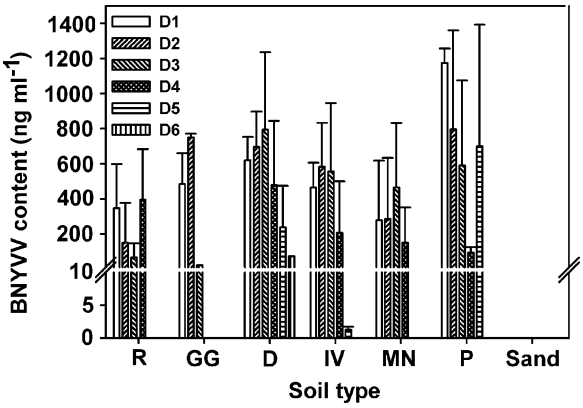

Fig. 1 Means of BNYVV concentration (ng ml^{-1}) in lateral roots of infected susceptible sugar beet cultivar as determined by quantitative DAS-ELISA. 10 plants per dilution were grown for 4 weeks in the climate chamber (16 h assimilation light, 24° C day— and 20°C night-temperature) in 6 dilutions (D1=5^{-1}; D2=5^{-2}; D3=5^{-3}; D4=5^{-4}; D5=5^{-5}; D6=5^{-6}) of soil samples from Italy (Rovigo = *R*); Germany (Groß Gerau = *GG*); Spain (Daimiel = *D*); USA (Imperial Valley = *IV* and Minnesota = *MN*); France (Pithiviers = *P*) and an autoclaved sand control. Data represent two independent repetitions of the assay. Vertical bars represent standard deviation

BNYVV levels in *Rz1+Rz2* plants. Detailed results, including the number of plants infected in various dilution treatments, are displayed in Table 2. The data indicate that even in the lowest dilution of infested soil (dilution ratio 5^{-1}) R- and GG-soil produced infections in *Rz1+Rz2* plants with low virus titre (0.3 and 5 ng ml^{-1}, respectively) (Fig. 2). In contrast, D-, IV-, MN- and P-type soil produced much higher BNYVV concentrations in lateral roots (99, 33, 242 and 205 ng ml^{-1}, respectively), which were comparable to virus titres in susceptible plants grown in R– or GG-type soil.

Remarkably, different soils produced high virus concentrations in *Rz1+Rz2* sugar beet plants even at higher dilutions, although virus concentration tended to decline. D-, IV- and MN-type soil samples were still able to infect three out of 10 double-resistant plants at a dilution of 5^{-4}, and D-type soil even infected one plant in soil diluted 5^{-5}. In the P-soil sample analysed here 4/10 plants tested positive for BNYVV infection in soil diluted 5^{-3}. By calculating the difference between the MPN of susceptible sugar beets (MPN_{sus} Table 1) and BNYVV units g^{-1} soil overcoming resistance in the MPN test with the *Rz1+Rz2* genotype (MPN_{diff} Table 2), and then dividing the MPN_{sus} by the MPN_{diff}, it was possible to calculate the concentration of the resistance-breaking isolates necessary to cause significant virus concentrations in *Rz1+Rz2* double-resistant plants. Thus, D-soil samples still infected *Rz1+Rz2* plants at a concentration of about 20 BNYVV iu g^{-1} soil diluted 5^{-5}, IV-soil at 8 iu g^{-1} and MN-soil at 13 iu g^{-1} in soil diluted 5^{-4}. In P-soil, *Rz1+Rz2* plants still tested positive at 9.4 iu g^{-1} in soil diluted 5^{-3}. Although, MN— and R-soils displayed similar BNYVV densi-

Table 2 BNYVV concentration in soils from different geographic origins, determined with an *Rz1 + Rz2* sugar beet cultivar and DAS-ELISA measurement after 4 weeks greenhouse cultivation to calculate the MPN by means of number of infected plants / number of harvested plants shown for each soil variant in different dilution steps

Soil origin						
Dilution	R	GG	D	IV	MN	P
5^{-1}	6*/10**	1/10	7/10	4/10	10/10	6/10
5^{-2}	2/10	1/9	8/10	5/10	5/10	5/10
5^{-3}	0/10	0/9	5/10	3/10	3/10	4/10
5^{-4}	0/10	0/10	3/10	3/10	3/10	0/10
5^{-5}	0/10	0/10	1/10	0/10	0/10	0/9
5^{-6}	0/10	0/10	0/10	0/10	0/10	0/10
BNYVV i.u. ovc. *Rz****	n.a.	n.a.	20 b*****	8 ab	13 b	9.4 ab

Dilution ratio = 5

*Number of infected sugar beets

**Number of single-harvested sugar beets

***Number of BNYVV infectious units necessary to overcome *Rz1+Rz2* resistance

****Means with the same letter are not significantly different (LSD 0.05); n.a. not applicable, numbers in bold represent lowest soil dilution step, where out-dilution was observed

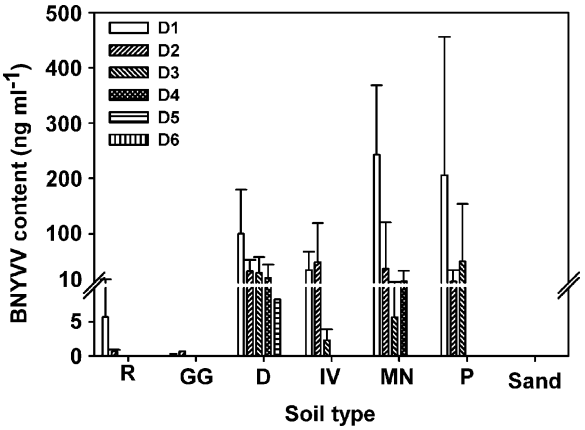

Fig. 2 Means of BNYVV concentration (ng ml^{-1}) in lateral roots of infected *Rz1+Rz2* partial-resistant cultivars determined by quantitative DAS-ELISA. Plants were grown for 4 weeks in the greenhouse in 6 dilutions (D1=5^{-1}; D2=5^{-2}; D3=5^{-3}; D4=5^{-4}; D5=5^{-5}; D6=5^{-6}) of soil samples from Italy (Rovigo = *R*); Germany (Groß Gerau = *GG*); Spain (Daimiel = *D*); USA (Imperial Valley = *IV* and Minnesota = *MN*); France (Pithiviers = *P*) and an autoclaved sand control. Vertical bars represent standard deviation

ties in the MPN test with susceptible plants, MN-derived BNYVV was able to overcome the resistance and successfully infected *Rz1+Rz2* plants. Similar results, however, were obtained with both soil samples IV and P. Both contained higher MPN values than MN and R and both were still able to infect *Rz1+Rz2*-resistant plants in soils diluted down to 5^{-3} and 5^{-4}, respectively, and produced elevated virus concentrations.

Determination of inoculum density and percentage of viruliferous *P. betae* in different resistance-breaking soils

As BNYVV inoculum concentrations in different soils did not correlate with resistance-breaking abilities, we investigated whether the virus vector inoculum density or the proportion of viruliferous *P. betae* of the population in the soil samples might be related to increased pathogenicity of the isolate. It was hypothesised that the *P. betae* concentration in soil should at least roughly correlate to the BNYVV MPN detected. To determine the MPN for *P. betae*, the rootlet saps used for BNYVV detection at 4 weeks harvest date were additionally used for relative *P. betae* quantification and subsequent estimation of *P. betae* concentration in soil. Relative *P. betae* concentrations were determined and compared to the different soil dilution treatments. In initial tests, *P. betae* zoospore densities in BNYVV-susceptible plants were compared with those in *Rz1+Rz2* plants. As expected, this did not result in significant differences, demonstrating that the BNYVV resistance in the sugar beet genotypes in this study did not influence the *P. betae* infection (data not shown). However, samples were taken from *Rz1+Rz2* plant roots only, as they suffered less from BNYVV infestation and displayed a healthy root system phenotype similar between the different treatments (Fig. 3).

Evaluating the TAS-ELISA results obtained, only in standard R-soil samples was *P. betae* out-diluted within the range of the serial dilutions applied. These observations are summarised in Table 3. A very low MPN value for infectious *P. betae* units was detected in the reference G soil sample. In contrast, D-soil seemed to possess very good conditions for *P. betae* multiplication in the field and displayed by far the highest MPN (8,900 iu g^{-1}), representing almost a 50-fold higher MPN than the standard R soil (180 iu g^{-1}) and even a 130 times higher MPN than the GG soil sample (67 iu g^{-1}). All resistance-breaking soils displayed higher *P. betae* concentrations than the reference soils R and GG.

Subsequently, the proportion of viruliferous zoospores in the soil samples analysed was calculated

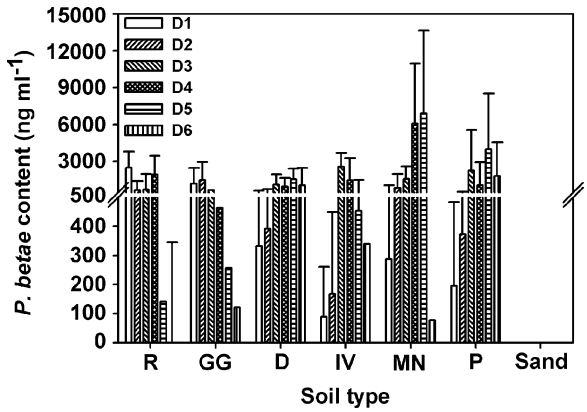

Fig. 3 Means of *Polymyxa betae* concentration (ng ml^{-1}) in lateral roots of infected BNYVV *Rz1+Rz2* partial-resistant plants determined by quantitative ELISA. Plants were grown 4 weeks in the greenhouse in 6 dilutions (D1=5^{-1}; D2=5^{-2}; D3=5^{-3}; D4=5^{-4}; D5=5^{-5}; D6=5^{-6}) of soils from Italy (Rovigo = *R*); Germany (Groß Gerau = *GG*); Spain (Daimiel = *D*); USA (Imperial Valley = *IV* and Minnesota = *MN*); France (Pithiviers = *P*) and an autoclaved sand control. Vertical bars represent standard deviation

Table 3 *Polymyxa betae* concentration in soils from different origins estimated by GST-specific TAS-ELISA based detection of zoospores from rootlets of an *Rz1* + *Rz2* sugar beet cultivar

Soil origin						
Dilution	R	GG	D	IV	MN	P
5^{-1}	10*/10**	10/10	10/10	10/10	10/10	10/10
5^{-2}	10/10	9/9	10/10	10/10	10/10	10/10
5^{-3}	6/10	1/9	10/10	9/10	10/10	10/10
5^{-4}	5/10	1/10	9/9	9/10	9/9	5/10
5^{-5}	1/10	1/10	10/10	4/10	7/10	4/9
5^{-6}	0/10	1/10	3/10	1/10	1/10	3/10
MPN g^{-1} soil	180 a****	67 b	8900 c	840 d	3300 e	960 d
% vir. *P. betae****	61%	16%	58%	61%	3%	35%

Dilution ratio = 5

*Number of infected sugar beets

**Number of single-harvested sugar beets

***Percentage of viruliferous *P. betae*

****Means with the same letter are not significantly different (LSD 0.05); letters in bold represent lowest soil dilution step, where out-dilution was observed

(Table 3). The MN-soil sample displayed a very low proportion of viruliferous zoospores (3%) compared to all other soils (16%–61%) and did not give any indication of a correlation of *P. betae* loading with the pathogenicity of the virus isolate.

Determination of *P. betae* reproduction abilities in different soils and soil dilution treatments

The quantitative *P. betae* TAS-ELISA allowed a determination of the *P. betae* multiplication rate in root hairs during the greenhouse experiment. *Polymyxa betae* possessed strong cystosory reproduction abilities during the four-week experiment under optimal temperature and humidity conditions, showing no clear or uniform relationship to soil origin and the initially adjusted inoculum density (Fig. 3). This was reflected in similar values of relative *Polymyxa* levels in different soil dilution steps, even demonstrating increased *P. betae* propagation *in planta* when low initial inoculum densities were applied. For example, the R-soil sample diluted 5^{-4}, had similar *P. betae* concentrations at the end of the four-week greenhouse experiment compared to no-diluted soil. In IV- MN- and possibly in P-soil samples, *P. betae* concentrations declined at the highest dilution step (5^{-6}) indicating the close out-dilution of the virus vector. The quite uniform concentration of *P. betae* over all dilution steps in D-soil and the lower multiplication of *P. betae* at the highest soil concentration (5^{-1}) in IV, MN and P-soils compared to R and GG soils (Fig. 3) was remarkable.

BNYVV resistance test with adjusted inoculum concentration

BNYVV and *P. betae* densities in naturally infested IV-, D-, MN-, and P-soils as well as the reproduction abilities in lateral roots did not correlate with the ability of soils to cause rhizomania symptoms in *Rz1* plants in the field (see above) or with the induction of elevated virus content in *Rz1*+*Rz2*-containing plants. To supply additional evidence that inoculum concentration and the ability to overcome resistance and produce severe virus symptoms did not correlate, a resistance test with adjusted inoculum concentration and three harvest dates (4, 8 and 12 weeks) was carried out. BNYVV inocula of 70 ng virus kg^{-1} soil consisting of dried sugar beet roots with each of the different rhizomania isolates was used. This concentration was averaged after determining the BNYVV concentration in lateral roots of a susceptible sugar beet cultivar grown in naturally infested soil (data not shown). Compared to resistance tests with field soil, both the 4 and 8 weeks test did not result in comparable virus concentrations in the lateral roots of susceptible plants in all treatments (data not shown). At 12 weeks, BNYVV DAS-ELISA in

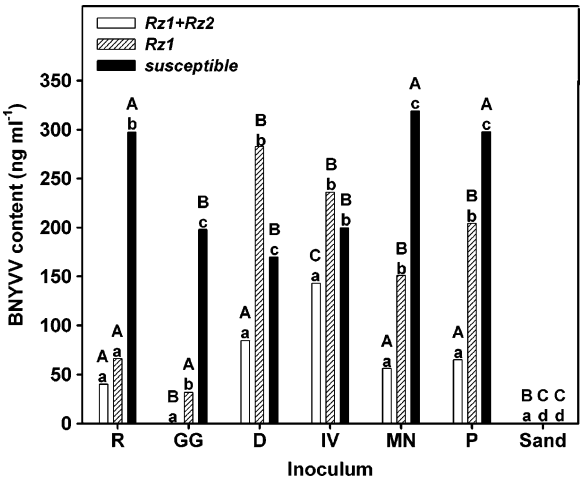

Fig. 4 BNYVV concentration (ng ml $^{-1}$) in lateral roots in a double-resistant (*Rz1+Rz2*) and single-resistant (*Rz1*) cultivar as well as in a susceptible control after 12 weeks greenhouse cultivation in artificially infested soil with different BNYVV isolates (from R-, GG-, D-, MN-, IV- and P-soil and a sand control). Means within the same soil type (lower case) and means within genotypes (capital) with a letter in common are not significantly different at the 5% level

general detected high virus concentrations in sugar beet lateral roots in plants of the susceptible genotype in all treatments except the sand control (Fig. 4).

Virus concentrations in lateral roots of *Rz1* plants differed significantly between treatments with inoculum from resistant-breaking isolates (D, IV, MN and P) compared to the two reference soils-derived isolates (R and GG). Resistance-breaking isolates produced virus concentrations between 160 to 280 ng ml^{-1} in *Rz1* rootlets, whereas R and GG induced only 40 and 65 ng ml^{-1}, respectively. For all but the D and IV soil type isolates, the virus concentration in *Rz1* cultivars was significantly reduced compared to susceptible cultivars (Fig. 4). For isolate D the *Rz1* cultivar displayed even higher virus concentrations than the susceptible genotype. In lateral roots of the *Rz1+Rz2* genotype, lower BNYVV concentrations were detected over all treatments; however, the differences between resistance-breaking and non-resistance-breaking soils were not as pronounced as observed in *Rz1* plants. Here resistance-breaking isolates produced BNYVV concentrations between 60 and 150 ng ml^{-1}. Remarkably, detectable virus concentrations of both reference isolates (R and GG) were observed in the *Rz1+Rz2* genotype. The values were slightly higher than those obtained in the MPN test.

In order to support the data obtained with quantitative ELISA, total plants and sugar beet taproots were weighed (Fig. 5). Noticeable were the high sugar beet weights of all treatments in *Rz1+Rz2* cultivars, compared to the other cultivars. No significant weight differences were evident between GG-, D-, IV-, MN- and P-isolates or sand control-grown taproots, but those infected with the R isolate had significantly higher weights compared to all other variants. In the *Rz1* treatments, taproot weights were strongly negatively correlated to BNYVV concentrations (data not shown). The higher the virus titre in lateral roots (Fig. 4), the more the taproot weights were reduced (Fig. 5). Taproot weight in *Rz1* sugar beet plants did not significantly differ between R-, GG-soil and the sand control but *Rz1* genotypes grown in D-, IV-, MN- and P-soils displayed significant weight reductions, and no significant differences compared to the susceptible genotype.

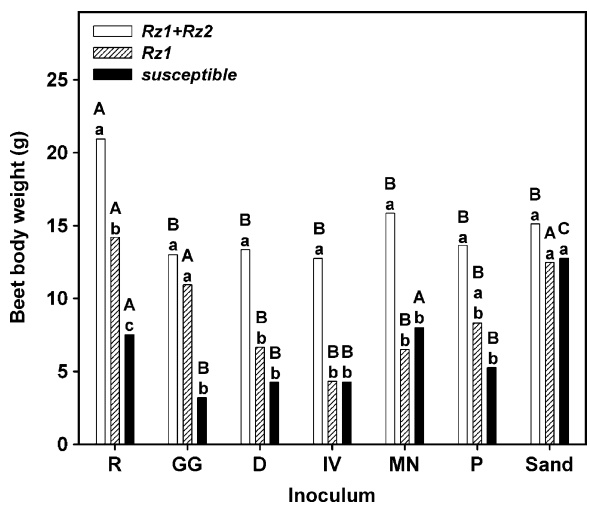

Fig. 5 Taproot weight (g) in double-(*Rz1+Rz2*) and single-(*Rz1*) resistant cultivars as well as in a susceptible control 12 weeks after greenhouse cultivation in sterilised soil artificially infested with lateral rootlets infected with different rhizomania isolates (from R-, GG-, D-, MN-, IV-, P-soils and a sand control). Means within the same soil type (lower case) and means within genotypes (capital) with a letter in common are not significantly different at the 5% level

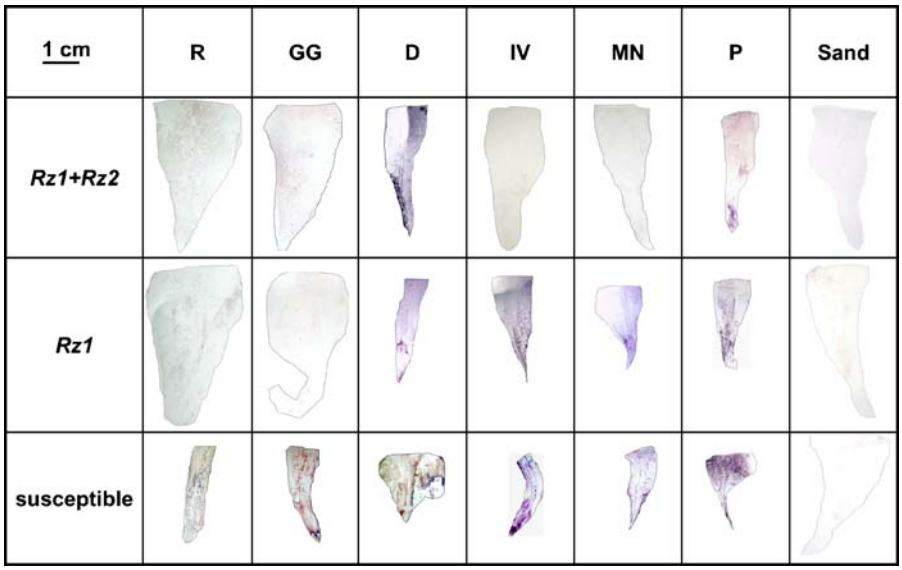

Fig. 6 Detection of systemic BNYVV spread in sugar beet taproots on membranes following TPIA in double-(*Rz1+Rz2*) and single-(*Rz1*) resistant cultivars as well as in a susceptible control after 12 weeks greenhouse cultivation in soil artificially infested with different BNYVV isolates (R, GG, D, MN, IV, P and a sand control). Dark bluish colouration represents BNYVV-specific antibody binding and shows the virus distribution in the taproot beet tissue

Systemic spread of BNYVV in taproots

Although the BNYVV resistance mechanism in sugar beet is not clearly understood, resistant plants are known to confine the virus to the rootlets and limit the spread to the taproot (Heijbroek et al. 1999; Scholten et al. 1994). Therefore, we wanted to investigate whether infection of lateral roots in both resistant genotypes with isolates from soil samples displaying resistance-breaking abilities led to isolate-dependent systemic virus spread in sugar beet roots. Hence, a TPIA of longitudinal beet sections was carried out (Fig. 6). Blue staining, indicative for BNYVV-specific antibody binding, demonstrated systemic but uneven spread of BNYVV in susceptible plants grown in all BNYVV-infected soils. As expected, only partial systemic virus infection with uneven distribution in the taproot was detectable in *Rz1*-resistant plants grown in D-, IV- MN- and P-soil. *Rz1* and *Rz1+Rz2* taproot tissue prints of GG and R treatments did not display any detectable substrate reaction. Remarkably, a systemic BNYVV spread in taproots of the *Rz1+Rz2* cultivar was only detectable in D- and P-soil-grown plants. Despite *Rz1+Rz2* lateral roots containing significant levels of IV and MN-derived isolates as shown above, no colour reaction above the background was observed in any plants tested in this assay.

Discussion

In this study rhizomania-infested soil samples from fields where BNYVV resistance-breaking strains of the virus were observed (Heijbroek et al. 1999; Liu et al. 2005; Liu and Lewellen 2007 and J. Ayala, personal communication) have been assayed for the first time in a growth chamber study under controlled conditions for BNYVV and *P. betae* inoculum concentration as well as for *P. betae* reproduction abilities in lateral roots. All samples were previously tested in parallel in 12-week greenhouse resistance tests and had been shown to induce BNYVV concentrations comparable to susceptible plants in *Rz1* cultivars (Pferdmenges et al. 2008). Additionally all A-type containing soil samples (D, IV and MN) produced detectable virus concentration in the *Rz1+Rz2* cultivar. The results of our study indicated that the resistance-breaking ability in the field and the capacity to produce elevated virus concentrations in

lateral roots of *Rz1* and *Rz1+Rz2* plants do not correlate with inoculum concentration and vector density in the soil samples analysed. This conclusion is supported by the results of several experimental approaches which are discussed below.

MPN with susceptible plants

The BNYVV MPN values calculated for the R and GG reference soil samples were in the range that Tuitert (1990) detected by comparing different MPN calculation methods using one Dutch soil harbouring an unknown BNYVV-type (40–100 iu g^{-1}). However, different MPN tests are known to be difficult to compare due to different calculation methods. Remarkably, even at this relatively low BNYVV MPN of approximately 100 iu g^{-1}, this was sufficient, in our study, to infect susceptible sugar beet plants in up to 5^{-4} diluted soil as shown for R and MN. The determination of the BNYVV MPN values revealed soil samples with similar MPN scores (i.e. MN and R) but differences in the aggressiveness of the virus. This observation gave the first indication that rhizomania pathogenicity did not correlate well to inoculum concentration. Overall, the BNYVV MPN-values obtained demonstrated a high variability in BNYVV concentration in the soil samples from different origins and that most of those that displayed resistance-breaking abilities under controlled conditions (D-, IV- and P- but not MN-soil) possessed a higher inoculum concentration than the reference soils displaying normal aggressiveness. It remains speculation whether elevated inoculum concentrations observed in most of the soil samples might be the cause for or the consequence of the increased aggressiveness.

BNYVV concentrations in lateral roots in relation to soil dilution

The high variability of virus concentrations in lateral roots measured in the whole study makes statistical analysis difficult but is inevitable if sugar beet hybrids are tested. Hybrids were used in this study to relate our findings to practical field observations. BNYVV-resistant genetically homogenous breeding lines, often representing one parent of the sugar beet hybrid, in general display much stronger resistance than cultivars (unpublished observations), but unfortunately are not suitable material to answer the question if resistance-breaking occurs under practical conditions or not. More important, however, is the finding that the virus concentration in all treatments did not decline in a linear manner with increasing soil dilution. There was no correlation in the reduction of the average virus concentration in relation to virus concentration in soil in the serial dilution steps as shown in Table 1. This may be explained by the potential of *P. betae* to multiply and produce similar numbers of secondary virus infection sites, independent of the initial inoculum density (shown in Fig. 3) efficiently starting infections in high soil dilutions from only few zoospore-releasing cystosori. On the other hand, it is possible that systemic virus infection in lateral roots in susceptible plants is not dependent on secondary vector infections. This is supported by systemic root infections following mechanical inoculation of 8 day-old BNYVV-susceptible seedlings as reported by Koenig and Stein (1990).

MPN with *Rz1+Rz2* resistant plants

Despite the D-soil sample displaying an extraordinary high MPN-value, the inoculum density should be excluded as the only factor responsible for resistance-breaking as plants of the *Rz1+Rz2* cultivar were still infected with BNYVV in soil diluted to 5^{-5}. Although the inoculum density of the MN and P soil samples was two and three orders of magnitude lower, respectively, than of the D-sample, *Rz1+Rz2* plants showed BNYVV infection in the 5^{-4} and 5^{-3} diluted soil (Table 2 and Fig. 2). This finding is also correlated to the average virus concentration observed in the MPN test with susceptible cultivars which was not strongly influenced by the inoculum density. We therefore conclude that inoculum concentration does not play a significant role for infection of *Rz1+Rz2* cultivars with BNYVV isolates displaying increased aggressiveness.

Polymyxa betae MPN of different soil samples and reproduction abilities in different genotypes

The *P. betae* MPN-values detected were elevated in all resistance-breaking soil samples, compared to the two reference soils, but the percentage of viruliferous *P. betae* did not correlate with virus resistance-breaking abilities. For example, the MN soil was

shown to overcome resistance but displayed only a low percentage of viruliferous *P. betae* in the soil. In contrast, the R soil sample contained 61% viruliferous *P. betae* but was unable to overcome resistance. Successful BNYVV transmission by its vector *P. betae*, which might be connected to the overcoming of resistance, may depend on genetical or ecological factors, but unfortunately information on *P. betae* variability concerning virus vectoring ability is still lacking.

In general, an unexpectedly high variation in the proportion of viruliferous *P. betae* was detected in this assay, ranging from 3–61%. The only published study where *P. betae* MPN-values were determined (Tuitert 1990) resulted in an estimation of only 10%–15% viruliferous *P. betae* in the soil. Due to the unequal distribution of *P. betae* in field soils, in general the MPN-values calculated are only representative for the soil samples analysed; these studies suggest much higher proportions of viruliferous *P. betae* in field soils than previously anticipated, despite there being no correlation between resistance-breaking and percentage of viruliferous *P. betae*. Our results also contrast to the findings of Gerik and Duffus (1988) who reported that viruliferous *P. betae* multiplied much more efficiently when an indigenous avirulent population was already present in the soil compared to soil free of *P. betae.*

Evaluation of the *P. betae* reproduction abilities in relation to soil sample origin, soil dilution and plant genotype, did not show a clear and consistent picture. However, the most relevant finding was that independent of the sugar beet genotype and soil origin, strong *P. betae* reproduction was observed in several high soil dilution steps. Assuming virus transmission during *P. betae* reproduction and secondary site infections in the *Rz1+Rz2* cultivar during the 4-week MPN-experiment, the conclusion that no correlation between virus inoculum density in the soil and resistance-breaking was detectable in this study is well supported. Despite the *P. betae* inoculum density in IV, MN and P soil samples being higher than the reference soils (Table 3), at the lowest dilution, only limited propagation was observed (Fig. 3). This might be explained by concentration-dependent self-inhibition of zoospore release from long-resting cystosori, as in higher soil dilutions, *P. betae* propagated to a level comparable to other soils with overall lower vector density; however, experimental evidence is lacking. In addition, an effect of other soil-borne pathogens naturally occurring in soil may play an important role in the virus-vector life cycle as they might interfere with BNYVV uptake and *P. betae* infection. Taken together, in this study there was no indication found for an influence of the viruliferous vector concentration and reproduction on the resistance-breaking abilities of the virus.

Resistance test with adjusted inoculum

The resistance test using adjusted inoculum levels was finally performed to minimise possible effects of inoculum density on virus aggressiveness. In addition, possible soil structure effects on *P. betae* propagation were excluded and detrimental effects of other soil-borne fungi minimised with soil sterilisation. Unfortunately systemic virus infection in susceptible plants commencing from dried lateral root inoculum took longer compared to infection in natural soil, leading to consistent infection of the susceptible control plants only after 12 weeks. This might be explained by an inhibition of zoospore release from freshly dried roots added to sterilised soil, due to a possible dormancy phase or inhibiting soil compounds compared to infestation of viruliferous *P. betae* in naturally infested soil. However, the differences in virus concentrations measured in lateral roots between the different genotypes, which indicates resistance breakdown, were in good agreement with the data obtained in naturally infested field soil (Pferdmenges et al. 2008). Of note is that resistance-breaking isolates D and IV produced lower virus contents in rootlets of susceptible plants compared to the other isolates. This may be explained by the detrimental effect on the taproot and less overall root growth, reducing the fitness of host cells or the amount of plant tissue available for virus replication.

Clearly the corresponding BNYVV concentrations of the different isolates produced in the *Rz1* plants in both assays, strongly supports the resistance-breaking abilities of D, IV, MN and P. The low virus concentrations in rootlets of the *Rz1* genotype grown in R and GG corresponded to previous observations by Heijbroek et al. (1999), who compared another *Rz1* sugar beet genotype in resistance tests (in this study the ELISA tests were performed after 8 weeks) in soil samples from the same geographic areas as R, GG and P soils;

similarly a low virus concentration in the R and GG treatments was detected. In general, the virus concentrations in lateral roots of the *Rz1* cultivar were higher than those in the double-resistant cultivar, reflecting the known additive effects of the two major resistance genes in greenhouse experiments and field resistance tests (Gidner et al. 2005; Liu et al. 2005). The Spanish soil-derived isolate (D) induced similar virus concentrations in *Rz1* plants compared to all other isolates known to overcome *Rz1* in the field and greenhouse (Liu et al. 2005; Liu and Lewellen 2007).

The virus concentrations of the different isolates detected in *Rz1+Rz2* plants in this study, however, slightly differed from those obtained in the 12-week greenhouse resistance test using natural field soil (Pferdmenges et al. 2008). In addition to D-, IV- and MN-, the P-isolate produced elevated BNYVV contents in rootlets of double-resistant plants. Overall, with resistance-breaking isolates, higher BNYVV concentrations in *Rz1+Rz2* plants were produced when compared to the previous resistance test. However, it remains unexplained why the reference R-isolate was able to induce detectable virus concentrations in double-resistant plants; a possible reason is the variability of the assay (growth chamber vs. greenhouse) or the genetic variability of the cultivar. It is known from previous studies that resistance performance is negatively influenced by increasing mean temperatures (Blunt et al. 1991), and we speculate that higher temperatures in the greenhouse might have compromised the quantitative double-resistance, leading to detectable levels of the P- and R-isolates.

The taproot weight analysis of *Rz1* plants reflected the ability of the different isolates to overcome resistance. However, our results are in contrast to previous findings of Heijbroek et al. (1999) who did not detect root weight differences with R-, GG- and P-soil-derived isolates. This might be explained by differences in the experimental design such as soil sample origin and/or greenhouse cultivation time.

Despite elevated BNYVV concentrations of the more aggressive strains in *Rz1+Rz2* plants, the weight of the beets were similar in all treatments, over the 12-week cultivation time. This double-resistance may hold up in the field against more aggressive strains as observed by Liu and Lewellen (2007) for IV and MN. The TPIA-mediated detection of systemically infected (although not evenly distributed) sugar beet taproots in the susceptible genotype was according to our expectations and previous studies (Kaufmann et al. 1992). The systemic taproot infection of *Rz1*-plants only in treatments with the more aggressive isolates is consistent with the high virus concentrations in rootlets and adds to the growing body of evidence that *Rz1* resistance is compromised in these soil origins. The TPIA analysis of *Rz1+Rz2* taproots did not show results consistent with the BNYVV concentration measured in rootlets. As virus contents were significantly lower compared to *Rz1* plants and beet weight was not affected, no systemic infection was expected. As D and P isolates systemically infected double-resistant beets, despite not producing BNYVV concentrations as in susceptible rootlets, the following conclusions are possible. Either D and P isolates overcome *Rz1+Rz2* by a different mechanism to IV and MN isolates or the systemic infection by IV and MN isolates in this double-resistant cultivar occurs too infrequently to be detectable with the limited number of 10 plants analysed here. The possibility remains that BNYVV concentrations in IV and MN-infected *Rz1+Rz2*-infected taproots were below the detection limit of the TPIA. The finding that high virus concentrations in rootlets are not necessarily connected to systemic infections is important. Further field and greenhouse experiments are needed to characterise the relationship of virus content in rootlets, systemic infection of the taproot and the induction of rhizomania symptoms with yield reduction in compatible and incompatible virus-host interactions, in order to find an appropriate phenotypic marker for the resistance-breaking phenomenon.

Taken together, these findings give additional evidence for the conclusion already drawn from the MPN-assay with both susceptible and resistant plants, that virus inoculum density is not responsible for increased aggressiveness of several BNYVV isolates from geographically separated regions (including one isolate that possesses an additional RNA 5) which probably have evolved independently. If the inoculum density in soil can be excluded as the main factor for resistance-breaking, other soil-borne synergistically-acting pathogens and virus mutation remain as possible reasons. Soil-borne fungi which have been detected in the different field soils of this study (Pferdmenges et al. 2008) cannot be excluded but their influence was at least reduced in the resistance

test with adjusted inoculum. Unfortunately, discussions about resistance-breaking mutations in BNYVV are still speculative despite the P25- and P26-encoding ORFs being the most often cited candidates. P25 represents the viral pathogenicity factor responsible for the development of root beard-like symptoms and yield reduction (Koenig et al. 1991) and possesses highly variable amino acid motifs (Schirmer et al. 2005). Additionally, phenotypic local lesions on mechanically-inoculated sugar beet leaves are dependent on P25 composition, and vary greatly between different BNYVV isolates and resistance sources (Tamada et al. 1989; Chiba et al. 2008). Therefore, mutations in P25 are plausible candidates responsible for increased BNYVV pathogenicity, though experimental evidence is lacking. As Tamada et al. (1996) reported that RNA 5-encoded P26 in addition to P25 is responsible for symptom development in sugar beet roots, and Link et al. (2005) proved P26 pathogenicity in *C. quinoa*, a synergism might be assumed. However, additional experimental approaches using viral full-length clones (Quillet et al. 1989) to perform site-directed mutagenesis and mechanical inoculation of sugar beet (Koenig and Stein 1990), to exclude the possible vector influence on pathogenicity, are necessary to identify the viral mutations responsible for this observed increased virus pathogenicity.

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