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Identification of rhizomania-infected soil in Europe able to overcome *Rz1* resistance in sugar beet and comparison with other resistance-breaking soils from different geographic origins

Friederike Pferdmenges · Helmut Korf · Mark Varrelmann

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Abstract Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV), is vectored by *Polymyxa betae*. The disease can only be controlled by growing partially resistant sugar beets, which quantitatively reduce virus replication and spread. None of the known major resistance genes (*Rz1*, *Rz2*, *Rz3*), alone or in combination, are able to prevent BNYVV infection entirely. Here we report for the first time the identification of a Spanish soil, containing an A-type BNYVV with RNA 1-4, displaying *Rz1* resistance-breaking abilities comparable to soils from the USA and to those from France containing the French (Pithiviers) P-type BNYVV with RNA 5. A resistance test with several soil samples vs. different sugar beet cultivars was conducted under standardised conditions. Sugar beets were analysed after 12 weeks of greenhouse cultivation for taproot weight, BNYVV and relative *P. betae* content. The soil samples from Spain, France and the USA produced high virus contents and strong rhizomania symptoms in *Rz1* plants, indicative of resistance-breaking abilities. In addition, all resistance-breaking soil samples produced detectable virus concentrations in plant lateral roots of the *Rz1*+*Rz2* cultivar, and plants grown in the Spanish soil sample also had reduced taproot weight and displayed severe rhizomania disease symptoms. Additionally, the main pathogenicity factor P25, responsible for the formation of BNYVV symptoms, showed high sequence variability in the amino acid tetrad at position 67–70. The results suggest the geographically independent selection of BNYVV resistance-breaking isolates following the uniform cultivation of *Rz1*-containing sugar beet cultivars.

Keywords *Beta vulgaris* ssp. *vulgaris* · *Beet necrotic yellow vein virus* · BNYVV · *Polymyxa betae* · *Rz1* · Sugar beet

F. Pferdmenges · H. Korf · M. Varrelmann (✉)
Institute of Sugar Beet Research,
Holtenser Landstr. 77,
37079 Göttingen, Germany
e-mail: varrelmann@ifz-goettingen.de

Introduction

Rhizomania is one of the economically most important sugar beet diseases world-wide. *Beet necrotic yellow vein virus* (BNYVV) causing rhizomania was identified in 1958 in Italy as the causal agent for severe yield reductions in sugar beet. BNYVV and several other soil-borne viruses (*Beet soil-borne virus*, BSBV; *Beet soil-borne mosaic virus*, BSBMV; *Beet virus Q*, BVQ) are transmitted by the biotrophic plasmodiophorid *Polymyxa betae* (Keskin 1964; Abe and Tamada 1986; Wisler et al. 1994). While the vector itself hardly affects sugar beet growth (Rush 2003), severe symptoms on the foliage (pale leaves in an upright position with fluorescent necrotising yellow veins) and drastic reduction of taproot devel-

opment including extensive proliferated secondary rootlets with brownish discolouration of the vascular system are induced by the virus (Tamada 1999; Scholten and Lange 2000). In recent decades, BNYVV has spread to all important sugar beet growing areas in Asia, USA and Europe (Asher et al. 2003; Tamada 1999; Lennefors et al. 2000; Nielsen et al. 2001). A permanent increase of rhizomania-infested fields in Europe is expected; Richard-Molard and Cariolle (2001) calculated an increase from 610,000 ha in 2000 to approximately 900,000 ha BNYVV-infected sugar beet fields in 2010.

BNYVV belongs to the genus *Benyvirus* and possesses a multipartite genome, with four or five components. The genome organisation and gene functions of each RNA segment have been reviewed by Tamada (1999). Molecular analysis of the BNYVV genome revealed three major types (Kruse et al. 1994; Koenig and Lennefors et al. 2000; Schirmer et al. 2005). The A-type occurs in Greece, former Yugoslavia, Slovakia, Austria, Italy, Spain, France, Belgium, the Netherlands, England, Turkey, Kazakhstan, China, Japan and the USA. BNYVV B-type occurs in Germany, in the upper Rhine valley, in France and in Switzerland. A and B types possess four RNA segments (Koenig et al. 1986). In Europe, BNYVV P-type occurs only in a small region near Pithiviers in France (Koenig et al. 1997) and in two sites in the UK (Ward et al. 2007). BNYVV-P is the only European type that contains an additional RNA 5. In Asia BNYVV isolates (BNYVV-J) with an additional RNA 5 were identified which display sequence variability to French P-type as well as to the classical A-and B-types (Koenig and Lennefors 2000; Schirmer et al. 2005). BNYVV-P, containing RNA 5, is characterised by its more rapid spread in plants (Heijbroek et al. 1999). Tamada et al. (1996) identified increased virulence of Asian RNA 5 containing BNYVV isolates in sugar beet roots. RNA 3-encoded P25 is highly variable, acts as the main pathogenicity factor and is responsible for BNYVV symptoms on sugar beet roots and development of severe chlorotic or yellow lesions on *Chenopodium quinoa* leaves (Koenig et al. 1991; Jupin et al. 1992). Local lesion types on mechanically inoculated sugar beet leaves depend on P25 sequence and vary greatly between different BNYVV isolates and resistance sources (Tamada et al. 1989 and Tamada 1999; Chiba et al. 2003). P25 displays high variability in a specific amino acid (aa) tetrad at position 67–70 ($aa_{67–70}$) which correlates with the BNYVV geographic origin (Schirmer et al. 2005). Link et al. (2005) observed that the RNA 5-encoded protein P26 serves as an additional pathogenicity protein.

Severe sugar beet yield losses can only be prevented by growing partially resistant genotypes. The first partially resistant sugar beets were developed in the mid 1980s. The cv. Rizor showed significant partial resistance and good yield improvements under rhizomania infection compared to yields in non-infested soil (De Biaggi 1987). Lewellen et al. (1987) reported partially resistant material from the Holly Sugar Company identified in field trials. The Holly source still contains the most important major dominant gene named *Rz1* (reviewed by Scholten and Lange 2000). Additional wild beet (WB) accessions served as further sources for the identification of other major resistance genes, such as *Rz2* and *Rz3* (Whitney 1989; Scholten et al. 1994 and 1999; Gidner et al. 2005). However, all known major resistance genes, alone or in combination, provide only partial resistance. *Rz1*, *Rz2* and *Rz3* only possess the ability to reduce the virus replication in hair roots and inhibit virus spread to the taproot (Heijbroek et al. 1999; Scholten et al. 1994; Tamada 1999). Thus, a permanent increase of BNYVV inoculum potential in soil seems to be inevitable, despite the cultivation of partially resistant sugar beets.

A-type isolates able to infect cultivars carrying *Rz1* as well as *Rz1*+*Rz2* resistance genes occurred in soils from the USA BNYVV (Liu et al. 2005; Liu and Lewellen 2007). Within these isolates a P25 aa motif with valine instead of alanine on position 67 was determined, but evidence of P25 $aa_{67–70}$ composition effect on pathogenicity in sugar beet has not so far been provided. In addition, the influence of the vector *P. betae* (density in soil, ability to transmit BNYVV, mobility in soil etc.) is unknown, although Tuitert (1990) has shown that at least in susceptible cultivars yield reduction and rhizomania symptoms are dependent on the presence and density of viruliferous *P. betae*.

Recently, in Spanish cultivar trials, severe BNYVV symptoms were observed in resistant cultivars (Julian Ayala, personal communication). To prove the resistance-breaking abilities of this soil, it was compared in a test comprising different sugar beet genotypes

with soils from different origins with known resistance-breaking abilities (Liu et al. 2005) under standardised greenhouse conditions. The BNYVV content in lateral roots of beets grown in these soils was quantified by ELISA. To study a possible effect of *P. betae*, its concentration in plants and sequence data for the ribosomal DNA internal transcribed spacer (ITS) region were determined. The ITS region (White et al. 1990) was used to assess the phylogenetic diversity to resolve the taxonomy of *P. betae* from different origins. To identify additional soil-borne pathogens possibly affecting sugar beet growth and BNYVV content in lateral hair roots by synergism with BNYVV, fungal isolates recovered from beets grown in the different soils analysed were identified at the genus level.

Materials and methods

Origin of soil samples, determination of BNYVV type and greenhouse conditions applied in resistance tests

Soil samples from Italy (Rovigo, referred to as R), Germany (Groβ-Gerau, GG), Spain (Daimiel, D), USA (Imperial Valley, IV and Minnesota, MN), France (Pithiviers, P), collected from different locations in Europe and the USA were evaluated in greenhouse resistance tests. GG and R soil samples were collected in fields where BNYVV-resistant cultivars were grown in standard 3-year rotations during the last two decades, and no BNYVV symptoms or yield reductions had been observed to date. All other soil samples were collected from fields where cultivars carrying the Holly/*Rz1* resistance displayed typical rhizomania symptoms. The samples were collected when symptomatic BNYVV-resistant sugar beets were still in the field [personal communication B.L. Lennefors (Syngenta Seeds, Sweden), J. Ayala (AIMCRA, Spain) and R.T. Lewellen (USDA, Salinas CA)]. Soil-samples were homogenised, air-dried and kept at room temperature until use. The presence of different BNYVV-types was verified in hair-roots of sugar beet bait plants (BNYVV-susceptible seedlings grown for 4 weeks) by RT-PCR amplification and sequencing of the RNA 3-encoded P25 ORF.

For greenhouse resistance tests, all soil samples and an autoclaved sand control were diluted in equal parts with autoclaved sand; 700 ml plastic pots were filled with 100 ml clay granulate for drainage and topped up with a 650 g soil sample. To avoid contamination between different soils, pots were arranged according to the soil sample in disinfected plastic containers. Within containers, all samples were randomised in a complete block design with ten replications per genotype vs. soil origin. Soil sample containers were placed at least 30 cm apart to avoid contamination by splashing during irrigation. Seven day-old seedlings, previously sown in sterile sand, were transplanted into fully saturated soil. Seven days after planting, the beets were sprayed with fungicides (0.2 g l^{-1} Tachigaren 70®, Bayer, Germany) to suppress stem damping-off due to *Aphanomyces* spp., *Pythium* spp. and *Fusarium* sp. The climate chamber was maintained at 23°C during daytime and 20°C at night, with a 16 h photoperiod.

Plant material

Sugar beet cultivars and lines in greenhouse resistance tests included a BNYVV-double resistant cultivar (*Rz1rz1+Rz2rz2*, referred to as *Rz1+Rz2*), a single-resistant cultivar (*Rz1rz1*, referred to as *Rz1*) and a susceptible sugar beet line (*rz1rz1*) as control. The plant material was the same as used by Liu et al. (2005) and Liu and Lewellen (2007) to demonstrate the resistance-breaking abilities of IV and MN soil samples. The *Rz1rz1* resistance, also known as the Holly source (Lewellen et al. 1987) was selected from *B. vulgaris* subsp. *vulgaris*, whereas *Rz2rz2* originated from the *B. vulgaris* subsp. *maritima* accession WB42 (Whitney 1989).

BNYVV RT-PCR detection and P25 sequencing

Total RNA was extracted from sugar beet lateral roots using the RNeasy kit (Qiagen, Hilden, Germany) and used for RT-PCR amplification of RNA 3-encoded P25 RNA. Primers P25-up (5′-TCGGAATATCCATT TAAAAG-3′) and P25-low (5′-GTCCCAACCAGAT CAACAA-3′) designed on BNYVV RNA 3 B-type sequence (Acc. no. M36894) amplified a 906 bp fragment (nt. 302–1207) from all types. The following PCR programme was conducted: 96°C for 2 min, 36 cycles of 96°C for 45 s, 50°C for 45 s and 72°C for 1 min and final synthesis for 10 min 72°C. For BNYVV detection, PCR-products were visualised on

agarose gels and sequenced without further cloning to detect possible mixed infections of BNYVV with RNA 3-encoded P25 sequence variants.

Rating of rhizomania symptoms, BNYVV ELISA-based detection and quantification

After 12 weeks of greenhouse cultivation sugar beets were harvested individually. Beets were carefully removed from the pot, and root-adhering soil was thoroughly washed away with water. Sugar beets were scored for BNYVV symptoms (yellowish leaf veins and dark brown lateral roots), before hair roots were separated from the beet body with a knife and dried on paper towels. Leaves were cut off below the hypocotyledon and discarded. Fresh beet bodies were weighed individually; shape and discolouration were scored on a linear scale from 0–9 covering the complete range of taproot stunting and presence of root beard over the whole experiment. The complete absence of BNYVV-symptoms was recorded as 0 whereas 9 represented a heavily BNYVV-infected plant displaying typical severe rhizomania symptoms (a small T-like taproot with brownish vasculature and dark brown lateral roots, as well as yellowing of leaf veins).

Quantitative DAS-ELISA was conducted following the method described by Gidner et al. (2005), with some modifications. Plant sap from lateral roots of each plant was obtained with a Pollähne leaf juice press (MEKU GmbH) and diluted in PBS-Tween-PVP at a ratio of 1:15. Each ELISA plate (Nunc A/S, Roskilde Denmark) with the exception of blanks and buffer-controls contained the following sample allocation: two samples each of healthy and infected plant sap, respectively, and a dilution series of purified BNYVV (Koenig et al. 1984). The dilution series, used to develop a standard curve, was based on twofold dilution steps between 2 and 4,000 ng ml^{-1} virus protein buffer and 0 ng ml^{-1}. To enable comparability between subsequent tests, aliquots of the standard (4,000 ng ml^{-1}) were kept at −20°C until further processing. BNYVV-specific antisera with similar sensitivity were obtained from Loewe (Sauerlach, Germany) and DSMZ (Braunschweig, Germany) and used in DAS-ELISA following the manufacturers' instructions. The absorption ($A_{405\ nm}$) of the colour reaction was measured after 1 h incubation at 37°C using a Titertek Multiskan Plus photometer (Magarete Malar, Nauheim, Germany). Samples were considered positive if they exceeded the mean plus three standard deviations of the healthy controls.

Quantification of *Polymyxa betae* zoospores

The quantification of *P. betae* by TAS-ELISA by specific detection of plasmodiophorid glutathione-*S*-transferase (GST) was carried out according to Kingsnorth et al. (2003) with some modifications, using the same lateral root samples as for the BNYVV quantification. All incubation steps of the *P. betae* TAS-ELISA were performed at 37°C for 1 h. The anti-rabbit antibody (IgG whole molecule, Sigma–Aldrich, Munich, Germany) was used as the alkaline phosphatase tagged antibody. For relative *P. betae* quantification of expressed GST, a purified GST (described in Kingsnorth et al. (2003) and kindly supplied by Broom's Barn Research Station, UK) was used. Purified GST, as a two-fold dilution series (1/1 to 1:2,048) was analysed in parallel by ELISA. The highest absorption value ($abs_{405\ nm}$ 1.2, 1/1) corresponded to a relative GST concentration of $_{log}3{,}000$ and the lowest absorption value ($abs_{405\ nm}$ 0.007, 1/2,048 dilution) displayed a relative GST concentration of $_{log}0$.

Detection and identification of *Polymyxa betae*

The DNA extraction from dried lateral roots grown in each soil was done following Liu et al. (2000) with slight modifications. For DNA extraction, lateral roots (1 g) were N_2-liquid frozen and ground to a fine powder in lysis buffer (400 mM Tris-HCl (pH 8.5), 60 mM EDTA (pH 8.5) 150 mM NaCl, 1% SDS) using mortar and pestle. All centrifugation steps were conducted at 11,000×*g* for 5 min at 4°C. DNA was used for Polymyxa-specific nuclear ribosomal DNA amplification gave a fragment of 454 bp using primers Psp1 and Psp2rev (Legrève et al. 2003) (Psp1 (5′-TAG ACGCAGGTCATCAACCT-3′) and Psp2rev (5′-AGGGCTCTCGAAAGCGCAA-3′)). The PCR-products were cloned and sequenced using standard primers.

Identification of other soil-borne pathogens

To detect the presence of other soil-borne pathogens that might have influenced the sugar beet growth through secondary infection, tissue from the inner

core of sugar beet taproots displaying root rot symptoms was surface-sterilised with EtOH and incubated on potato dextrose agar (PDA, Roth, Karlsruhe, Germany) +150 mg l^{-1} Streptomycin, Applichem, Darmstadt, Germany). From each genotype vs. soil variant five beets were chosen to detect the presence of any additional soil-borne fungi. After 9 days the outgrown mycelia was assigned to fungal genera using microscopic analysis. To identify the isolates on a species level, total DNA extraction (DNeasy, Qiagen, Hilden, Germany) was carried out and extracts were subjected to PCR amplification of ribosomal DNA with ITS4 and NS7 primers (White et al. 1990). PCR-products were purified with SureClean (Bioline, Mannheim, Germany) following the manufacturers' protocol, prior to direct sequencing using the NS7 primer. Sequencing was carried out by MWG Biotech AG, Germany. Sequences were used for Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) of the nucleotide database to identify the fungal species.

Sequence analysis

DNA sequences of soil-borne pathogens from each soil sample were used to test for polymorphisms in a region that corresponded to ITS1+5.8S gene+ITS2. To perform multiple nucleotide sequence alignments, the CLUSTALX algorithm using the Kimura correction (Thompson et al. 1997) was applied. The phylogenetic tree was drawn using TreeView 1.5.2 software (http://taxonomy.zoology.gla.ac.uk). The *P. betae* sequence [GenBank Acc. no. Y12827 (Ward and Adams 1998)] was used for sequence comparison of *P. betae* from different geographical origins. Additional ITS sequences of several *Polymyxa graminis* subspecies and other plasmodiophorids were taken for sequence comparison [*P. graminis* f. sp. *temperata* (GenBank Acc. no. Y12824), *P. graminis* f. sp. *tropicalis* (GenBank Acc. no. Y12825), *P. graminis* f. sp. *colombiana* (GenBank Acc. no. AJ010424), *P. graminis* f. sp. tepida (GenBank Acc. no.Y12826), *Ligniera* sp. (GenBank Acc. no. AJ010425), *Plasmodiophora brassicae* (GenBank Acc. no. Y12831)].

Data analysis

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

Results

Identification of the BNYVV-type in the different soil samples and determination of the RNA 3-encoded P25 aa 67–70

To demonstrate that the soils samples used in this study contained the BNYVV types and P25 composition as described (Heijbroek et al. 1999; Liu et al. 2005; Liu and Lewellen 2007; Schirmer et al. 2005), all soil samples were initially used for a BNYVV bait plant test with seedlings of the *rz1rz1*-susceptible sugar beet line with subsequent RT-PCR amplification and sequencing of an RNA 3 fragment containing the P25 open reading frame. The P25 aa 67–70 were determined as R soil (AHHG), GG (AYHR), D (A/VCHG), IV (VLHG), MN (VCHG) and P (SYHG), and in P-soil samples the additional RNA 5 was detected using P26 open reading frame specific RT-PCR (data not shown).

Sugar beet weight and scoring for rhizomania symptoms

Greenhouse resistance tests were conducted to test different rhizomania-infested soils for their ability to infect resistant sugar beet cultivars, to produce virus symptoms and to affect plant growth. After 12 weeks cultivation, the freshly harvested taproots were weighed (Fig. 1) and scored for root discolouration due to infection with viruliferous *P. betae* (Table 1). An extension of the test period from 4 (data not shown) to 12 weeks led to more significant differences between the various genotypes. In all soils the susceptible genotype displayed significantly lower beet weights compared to the non-infected (sterile) sand control (Fig. 1). Sugar beets grown in soil from D especially showed severe weight reduction with an average weight (aw) of only 0.27 g, compared to IV 1.04, MN 0.75 and P 0.9 g, respectively. In contrast,

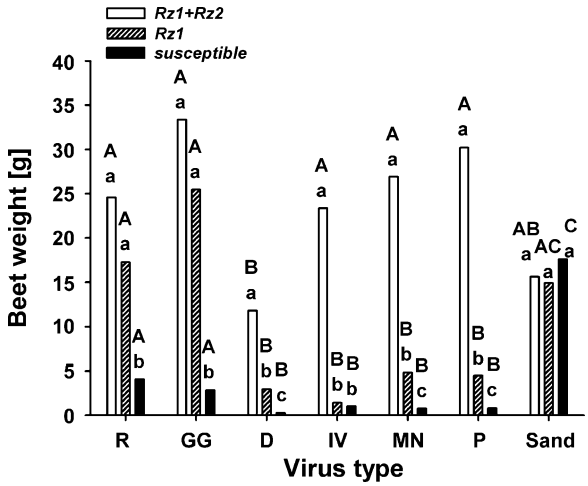

Fig. 1 Average beet weight from different sugar beet cultivars (*white*: *Rz1+Rz2*; *grey*: *Rz1* and *black*: susceptible control) after 12 weeks greenhouse cultivation in rhizomania-infested soils from *R* (Rovigo/Italy), *GG* (Groβ Gerau/Germany), *D* (Daimiel/Spain), *MN* (Minnesota/USA), *IV* (Imperial Valley/USA), *P* (Pithiviers/France) and an autoclaved *sand* control. Means within the same soil type (*lower case*) and means within genotypes (*upper case*) with a *letter in common* are not significantly different at the 5% level

soils from R (4.07 g) and GG (2.84 g) also influenced the plant growth significantly compared to the sand control (17.58 g) but not as strongly as the other soils in this study.

As expected, *Rz1* beets grown in R-and GG-soils were hardly affected by BNYVV. On the contrary, aw was greater in GG-soils (25.45 g) or similar to the susceptible control as in R-soils (aw 17.26 g) compared to the sand control (14.90 g). However, strong root weight reduction was observed for this cultivar in soils from D (2.93 g), IV (1.40 g), MN (4.81 g) and P (4.47 g). Furthermore, beet discolouration indicative of infection with fungal root rotting pathogens was observed, as in the susceptible genotype (data not shown). In comparison, plants of the *Rz1+Rz2* genotype displayed the highest aw for each soil beside sand (15.62 g). However, all genotypes in sand achieved similar weights. Remarkably, *Rz1+Rz2* beets grown in soils from R (24.58 g), GG (33.35 g), IV (23.39 g), MN (26.93 g) and P (30.21 g), obtained higher aw than plants grown in sterile sand. Only *Rz1+Rz2* sugar beets in D-soil gained less root weight (11.82 g) than plants grown in sterile sand and several beets showed discolouration and rotten tissue.

BNYVV symptom scores are displayed in Table 1. In general, infested beets did not show the ordinary beet shape, but developed a very small T-like phenotype instead. Frequently, leaves displayed typical yellow veins and brownish vascular system. The scoring of all genotypes in various soils was closely related to beet weight. All soils induced severe symptoms in the susceptible genotype (scoring 7–9). Again, D, IV, MN and P produced high scores in the *Rz1* genotype (scoring 6–9). In the *Rz1+Rz2* cultivar typical virus symptoms were displayed on plants grown in D, IV and marginally in MN. Remarkably, *Rhizoctonia solani* symptoms were identified especially on taproots of *Rz1+Rz2* and *Rz1* sugar beets cultivated in D soil.

Quantitative BNYVV contents measured by ELISA

Quantitative ELISA was conducted to measure the absolute BNYVV content in infested sugar beet hair roots as an attribute to resistance (Giunchedi et al. 1985, 1987; Bürcky and Büttner 1985). Results (Fig. 2) were negatively correlated to sugar beet weight. In all naturally infested soils, susceptible cultivars showed root weight reduction, extensive proliferation and secondary hair roots displayed necrosis and high virus content. In comparison to all soils analysed, P produced the highest average BNYVV-content (105 ng ml^{-1}) in lateral roots of the susceptible cultivar. In R-, GG-, IV-and MN-soils, BNYVV susceptible beets exhibited mean virus contents in hair-roots between 40 ng ml^{-1} (IV) and 74 ng ml^{-1} (R), whereas the same cultivar in D-soil

Table 1 Beet scoring for BNYVV symptoms (discolouration of the taproot, dark brownish hair roots, brown vascular, yellow leaf veins) on fresh harvested sugar beets (*Rz1+Rz2*, *Rz1* and susceptible) after 12 weeks greenhouse cultivation in naturally infested soil

Soil	*Rz1+Rz2*	*Rz1*	Susceptibility
R	0	0	7
GG	0	0	8
D	3	8	9
IV	2	7	9
MN	1	8	9
P	0	6	9
Sand	0	0	0

The scoring ranked from 0 (no infection) to 9 (fully infested sugar beet taproot)

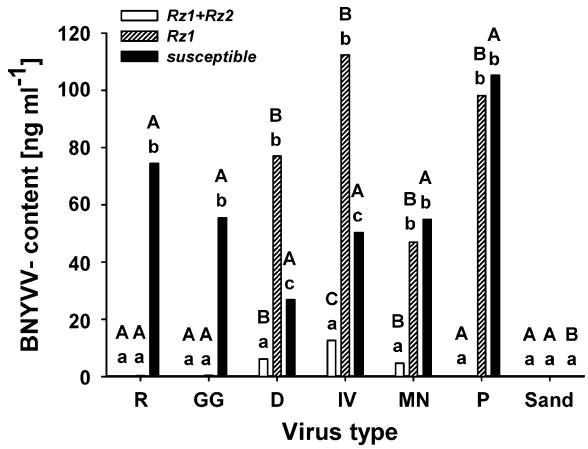

Fig. 2 Average BNYVV content (ng ml^{-1}) after quantitative ELISA from different sugar beet cultivars (*white*: *Rz1+Rz2*; *grey*: *Rz1* and *black*: susceptible control) after 12 weeks greenhouse cultivation in rhizomania-infested soils from *R* (Rovigo/Italy), *GG* (Groβ Gerau/Germany), *D* (Daimiel/Spain), *MN* (Minnesota/USA), *IV* (Imperial Valley/USA), *P* (Pithiviers/France) and an autoclaved *sand* control. Means within the same soil type (*lower case*) and means within genotypes (*upper case*) with a *letter in common* are not significantly different at the 5% level

displayed a significant lower average BNYVV content (27 ng ml^{-1}). In *Rz1* plants, the virus content was negatively related to the taproot weight, since only beets grown in D-, IV-, MN-and P-soils (from 47 ng ml^{-1} in MN to 112 ng ml^{-1} in IV soil sample) were heavily infected. In contrast *Rz1* plants grown in R- (mean virus content of 0.01 ng ml^{-1}) and GG-type (0.04 ng ml^{-1}) soil displayed negligible BNYVV concentrations.

Rz1+Rz2 plants in all infected soils displayed significantly lower virus content than either *Rz1* plants or the susceptible genotype. Soil from R and GG (both 0 ng ml^{-1}) differed greatly from the mean values of IV (13 ng ml^{-1}), D (6 ng ml^{-1}) and MN (5 ng ml^{-1}). In the P-soil treatment there was a negligible virus content of only 0.03 ng ml^{-1}. In resistance tests with 4 weeks cultivation time in D-, IV-, MN- and P-soils with an *Rz1+Rz2* genotype, BNYVV contents were observed to be ten to 20 times higher (data not shown), indicative of a recovery during the longer growth period. Sterile sand served as the control for both ELISA background and contamination between soils, and as anticipated, no virus was detected in plants of all three different genotypes (Table 1 and Fig. 2).

Relative quantification of *Polymyxa betae* via Polymyxa-specific GST ELISA

Evidence from previous studies suggests that enhanced viruliferous vector multiplication and BNYVV transmission might be involved in resistance-breaking (Scholten et al. 1994). A Polymyxa-specific GST ELISA was used to determine the concentration of zoospores, as an indication of the potential for *P. betae* to multiply. The ELISA revealed significant differences in *P. betae* propagation potential in the various soils analysed (Fig. 3). MN soil samples resulted in the highest *P. betae* concentration (from 1,747.44 to 4,018.24 GST relative units per milliliter root sap). D and P reached, on average, five to nine times lower *P. betae* GST protein content compared to MN. R and GG contained 16 times less *P. betae* in comparison to MN. However, a Polymyxa content within different soils depending on the sugar beet genotype was not evident.

Polymyxa betae variability-sequence and phylogenetic analysis

As *P. betae* isolates were derived from the same host, but from different regions worldwide, sequence variability may be connected to proximate adaptation

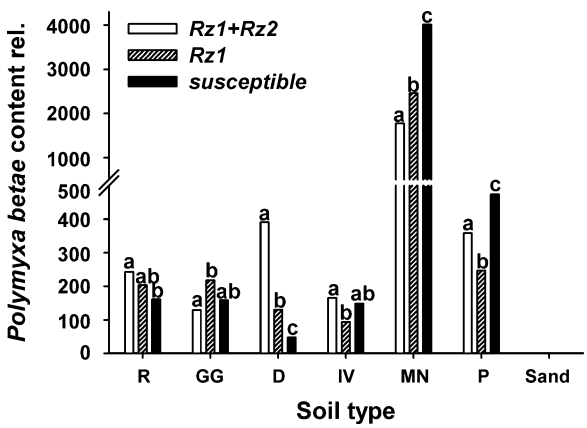

Fig. 3 Relative average P. betae content (ELISA-measured glutathione-S-transferase content) from different sugar beet genotypes (white: Rz1 + Rz2; grey: Rz1 and black: susceptible control) after 12 weeks greenhouse cultivation in rhizomania-infested soils from R (Rovigo/Italy), GG (Groβ Gerau/Germany), D (Daimiel/Spain), MN (Minnesota/USA), IV (Imperial Valley/USA), P (Pithiviers/France) and an autoclaved sand control. Means within the same soil type with a letter in common are not significantly different at the 5% level

to climatic conditions or other local factors. Genomic variability might influence virus transmission efficiency, the ability to multiply and the ability for long-term survival in soil. Clustal X alignment of sequences resulted in three different main branches which were assigned group 1–3 (Fig. 4). Group 1 contained *P. betae* isolates which originated from different soils in Europe and the USA. Remarkably, within this group there was a very high sequence homology. Only few single nucleotide exchanges were detected in the ITS1 +5.8S+ITS2 region, making the different *P. betae* isolates of this study phylogenetically very close. However, all *P. betae* isolates were distinctly separated from *P. graminis* (group 2). Within group 2, alignment resulted in clear separation of two subgroups (*P. graminis* f.sp. *temperate* and *P. graminis* f. sp. *tropicalis* and *P. graminis* f.sp. *colombiana* and *P. graminis* f.sp. *tepida*) after pairwise comparison. The third group comprised two other plasmodiophorid species (*Ligneria* sp. and *P. brassicae*).

Identification of other soil-borne pathogens

Although seed treatment was conducted to prevent damping-off diseases, an infestation with soil-borne pathogens was not entirely inhibited. The ITS region (ITS1+5.8S+ITS2) of extracted total DNA from fungal isolates growing out of surface-sterilised sugar beet taproot tissue were PCR-amplified. Subsequent to cloning and sequencing of the PCR product, the sequences were used for BLAST-search comparisons. The ITS1+5.8S+ITS2 sequences obtained from beets cultivated in R-and GG-soils were shown to originate mainly from *Fusarium oxysporum*. In Spanish D-soil, different pathogens were detected from several sugar beet pieces: *R. solani* (mainly in *Rz1+Rz2* and *Rz1* genotypes), *F. oxysporum* and *Pythium ultimum*. The US-soil MN and IV-derived soil samples contained different *Fusarium* species and *P. ultimum* similar to the D soil sample, but no *R. solani* was isolated. Additionally, several different *Fusarium* sp. isolates were detected in every sugar beet analysed in the French P-soil.

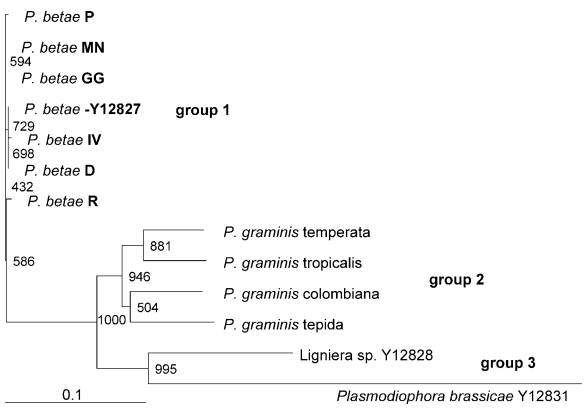

Fig. 4 Phylogenetic analysis of different *Plasmodiophoromycetes* based on rDNA ITS1+5.8S+ITS2 gene. The sequences were aligned and neighbour-joining trees were constructed using the CLUSTAL X programme. Bootstrap values were calculated from 1,000 replicates and are indicated at each node. The scale bar indicates 0.1 % substitution per bp. *P. betae* (GenBank Acc. no Y12827 and isolated from A-, B-, P-, D-, IV-and MN-soil samples of this study), *P. graminis* f. sp. *temperata* (GenBank Acc. no. Y12824), *P. graminis* f. sp. *tropicalis* (GenBank Acc. no. Y12825), *P. graminis* f. sp. *colombiana* (GenBank Acc. no. AJ010424), *P. graminis* f. sp. *tepida* (GenBank Acc. no. Y12826), *Ligniera* sp. (GenBank Acc. no. AJ010425), *Plasmodiophora brassicae* (GenBank Acc. no. Y12831)

Discussion

The occurrence of field soils containing aggressive BNYVV strains capable of causing reductions in root yield and quality in rhizomania-resistant cultivars has been reported from several sugar beet growing areas throughout the world (Tamada et al. 1996; Heijbroek et al. 1999; Liu et al. 2005; Liu and Lewellen 2007). The French P-type is assumed to exert its increased aggressiveness through its additional RNA 5 but in contrast the *Rz1* resistance-breaking isolates identified in the USA, possesses only four genomic RNAs. Here we report for the first time the occurrence of a European soil containing BNYVV with four RNAs and possessing similar resistance-breaking abilities to the isolates previously obtained from US fields in California and Minnesota. Moreover, this study describes the first comparative experiment on resistance-breaking abilities of BNYVV-infected soils under standardised greenhouse conditions in a resistance test. The results demonstrate that in independent areas where rhizomania-resistant sugar beet cultivars have been cultivated, resistance-breaking strains of the virus carrying four RNAs have evolved.

As BNYVV resistance is characterised by reduced virus titres in lateral roots of sugar beets (Bürcky and Büttner 1985), we measured virus concentration in hair-root sap to assess whether resistance-breaking

occurred. To obtain stronger evidence for resistance-breaking abilities of the different isolates, the cultivation time of identical genotypes was extended from 4 weeks, as applied in a previous study (Liu et al. 2005), to 12 weeks in a greenhouse resistance test. This enabled us to additionally observe an influence of virus concentration on root development, to evaluate whether the virus titre in hair-roots correlated with the degree of resistance, and whether symptomatic root development was induced.

For this study three different genotypes were examined in several soils, including those that were already known to produce severe symptoms on partially resistant sugar beets. As controls, two reference soil samples, a German BNYVV B-type (GG) and an Italian A-type containing soil (R), and a sterile sand control were applied. Samples from the same geographic region were previously included in the study of Heijbroek et al. (1999) and were shown there to exert only minor BNYVV effects on *Rz1* and Rizor cultivars. To assure the practical relevance of our results, instead of more uniform-reacting cultivars, homozygous breeding lines were chosen. The root yield of susceptible genotypes differed only marginally between treatments.

Although BNYVV isolated from the D-soil sample possessed only four RNAs, similar to the IV-and MN-isolates, increased pathogenicity represented by root weight and BNYVV content of infected *Rz1* plants was observed. For *Rz1*+*Rz2* plants, the highest reduction of taproot weight of all soil samples tested and an elevated virus concentration comparable to the IV and MN treatments was demonstrated by plants grown in D-soil. Detectable virus concentrations without strong root weight reductions were also measured in soil samples from the USA. Comparing differences in root weight of the sugar beet genotypes between the different soil samples, however, is not feasible, because weight differences might also be caused by varying nutrient concentrations in the samples.

A comparison of our quantitative virus data to previous observations (Liu et al. 2005; Liu and Lewellen 2007), that showed ELISA absorption values of IV and MN in both resistant hybrids at 4 weeks applying a seedling assay with 100 plants/pot, is difficult. However the virus concentration in the double-resistant cultivar grown in the IV and MN resistance-breaking soil samples in our 12-week assay is several orders of magnitude lower than in the *Rz1* plants, which is in disagreement to some previous studies that observed similar BNYVV ELISA absorptions in both genotypes. This might be explained by our extended test period, suggesting a possible time-dependent recovery from the infection. However Lennefors et al. (2006) detected differences in virus concentration between *Rz1* and *Rz1*+*Rz2* genotypes, in P-and B-type soil in 4-week tests, supporting our observations and suggesting additive effects of the resistance genes on the BNYVV concentration in lateral roots. This presumption is supported by Liu et al. (2005) who reported better performance of the double-resistant cultivar including higher sugar yield in IV soil in the field compared to the *Rz1* cultivar. When greenhouse tests for selection of BNYVV resistance were developed, selection time was shortened to 4 to 6 weeks in comparison to field tests, because lateral root virus concentration of sugar beet seedlings indicated the genotypic resistance potential even at that developmental stage (Bürcky and Büttner 1991). Therefore this time-span was used in most previous studies (e.g. Gidner et al. 2005; Lennefors et al. 2000; Lewellen et al. 1987; Paul et al. 1992; Scholten et al. 1994). In field resistance tests, however, the virus concentration in taproots remains the first priority because virus concentrations decrease in lateral roots throughout the vegetation period (Bürcky and Büttner 1991).

The D-isolate from the south of Spain characterised in this study belongs to the A-type (Schirmer et al. 2005). Since P25 represents the virus pathogenicity factor due to its impact on the development of root symptoms, the composition of the highly variable amino acid motif 67–70 (naturally present in 12 different combinations, Schirmer et al. 2005; Ward et al. 2007) is supposed to influence virus pathogenicity. The D-soil sample contained a mixed infection with two P25 variants possessing the tetrad composition VCHG and ACHG, and the USA-derived soil samples in this study both displayed P25 with V_{67}. Thus all RNA 1–4 containing resistance-breaking isolates in this study possessed V_{67} in the most variable motif of the virus pathogenicity factor (Schirmer et al. 2005). In contrast, Liu and Lewellen (2007) described soils within small geographical regions in the Imperial Valley and Minnesota district that contained BNYVV isolates possessing the aa V_{67} on P25, which were not able to overcome resistance in sugar beet, as well as BNYVV isolates expressing

varying amino acids on position 67, which produced severe rhizomania symptoms on partially resistant sugar beets.

Both reference soil samples were unable to overcome both *Rz1* and *Rz1+Rz2* resistance. Considerable BNYVV concentrations and taproot weight reductions were only observed in susceptible plants. Heijbroek et al. (1999) reported that GG seemed to be least pathogenic. This is also supported by our study although different genotypes (cultivars and lines) were used.

The P-soil sample produced the highest BNYVV concentration in hair-roots in susceptible plants and there was a remarkable reduction of *Rz1* plant root weight (similar to susceptible plants) in P-soil compared to the R-soil sample. These results are contrary to observations of Heijbroek et al. (1999), who detected strong tap-root weight reduction but no increased virus concentrations in hair-roots. This may be explained by the longer growth period applied and the different plant genotypes used. Although P-soil produced higher virus content in *Rz1+Rz2* plants than R-and GG-soil, root weight was not strongly reduced. The differences in beet weight of *Rz1* plants, however, corresponded well to the variations in BNYVV content measured in lateral roots. The French BNYVV P-type is phylogenetically classified as an A-type carrying an additional RNA 5 (Schirmer et al. 2005).

In addition to our observations, several studies reported P26 to be an additional BNYVV encoded pathogenicity factor (Tamada et al. 1996; Miyanishi et al. 1999). This suggests that RNA 3-encoded P25 pathogenicity factor and RNA 5-encoded P26 enable the virus to overcome *Rz1* in a synergistic fashion, allowing the virus to replicate and spread more efficiently. This assumption might explain also the much higher virus concentrations of the P-type in susceptible plants compared to the RNA 1–4 carrying resistance-breaking isolates. However, we did not observe resistance-breaking in P-type infected plants carrying *Rz1+Rz2* at 12 weeks. Whether only P26 or both pathogenicity factors are responsible for this effect remains elusive. Only pseudorecombinant isolates of different BNYVV-A-and P-type isolates and efficient mechanical inoculation techniques of sugar beet roots or *P. betae* loading will help to answer this hypothesis. The same experimental approaches including viral full-length clones will be necessary to answer whether P25 tetrad 67–70 is responsible for resistance-breaking. Further investigation is required to supply evidence on whether stable resistance is solely based on the presence of *Rz2* or due to a quantitative effect of both major genes.

Since all soils tested were used in the same dilution without determining and adjusting inoculum density, we cannot state if inoculum density had an influence on the resistance-breaking abilities of P-type and D-isolate as Scholten et al. (1994) reported previously. However, a prerequisite for successful BNYVV-transmission is a sufficient number of viruliferous *P. betae* zoospores in the soil (Asher et al. 2003) and all soils in the test were able to infect the susceptible control plants. Scholten et al. (1994) applied different numbers of viruliferous *P. betae* zoospores and suggested that the vector's ability to multiply in sugar beet roots plays a significant role regarding a possible resistance break. Using non-adjusted vector concentrations in soil, we compared *P. betae* concentration and its ability to multiply in hair-roots during the 12-week resistance test. Cultivars in R, GG and IV obviously did not differ significantly within the same soil, whereas plants grown in D, MN and P displayed variable *P. betae* contents. Regarding R, GG and IV, *P. betae* propagated to similar levels in susceptible and partially resistant cultivars; however *P. betae* augmentation was different in susceptible and partially resistant genotypes grown in D, MN and P. Overall, no relationship between *P. betae* propagation and quantitative resistance trait was recorded. However, the noticeable high *P. betae* $\text{content}_{\text{rel.}}$ in MN soils (on average ten times higher *P. betae* concentration than in all other measured soils) was indicative for quite effective *P. betae* propagation and/or high initial soil inoculum concentration.

The BNYVV-susceptible and the *Rz1* cultivar both displayed impressively low *P. betae* $\text{content}_{\text{rel}}$ (only a tenth of the content in the *Rz1+Rz2* plants) in D soil. It is suggested that the great deterioration of lateral and taproots due to BNYVV infestation after initial infection inhibit *P. betae* from emerging and propagating in cells surrounding the initial zoospore infection site (Kaufmann et al. 1992). Hence, the correlation of vector densities and virus content in lateral sugar beets as described by Asher et al. (2003) and Scholten et al. (1996) was not detectable in this study.

A previous demonstration of genomic diversity in *P. graminis* ITS sequences led to the proposal of subspecies, but no differences between two geographically distinct *P. betae* isolates (Belgium and Turkey) were observed (Legrève et al. 2002). In the present work, differences between *P. graminis* and *P. betae* in the ITS sequences were obvious, as well as diversity within the *P. graminis* subspecies, but all *P. betae* isolates, although originating from different areas worldwide, hardly differed in the analysed sequence. However, this does not exclude differences in the ability to multiply to take up and transmit BNYVV. Thus more detailed genetic analysis is necessary to unravel possible variability in these virus-vector properties.

Several beets displaying discolouration were tested for infestation by soil-borne fungi to see if there was a coincidence of fungal infection with severe tap-root weight reduction and virus concentration, and to detect a possible synergism with rhizomania from specific geographic origins. All sugar beets, independent of their genotype, were infested with soil-borne fungi. Ubiquitous *Pythium* was detected in most cases. Although the different *Fusarium* isolates were not tested for their pathogenicity and ability to produce symptoms and damage in sugar beet, no obvious relationship of *Fusarium* isolation to resistance-breaking abilities of rhizomania was observed. In contrast, *R. solani* was only recovered from symptom-carrying sugar beets grown in the D-type soil sample. As fungicide treatment was not effective, we cannot exclude a negative influence on the plants' BNYVV resistance abilities, but again a specific synergistic effect of *Rhizoctonia* with BNYVV resistance-breaking was not observed, as beets from other soils in this study did not contain this fungus at detectable levels.

Taken together this study demonstrated that (1) in addition to known soils from the USA and Pithiviers, *Rz1* resistance in sugar beet was compromised in a soil sample from Spain, containing BNYVV with only four components and a specific P25 tetrad composition (2) the vector *P. betae* from soils of different regions worldwide did not exhibit phylogenetic diversity in the ITS1+5.8S+ITS2 rDNA region (3) the vector's propagation ability in hair-roots showed a strong variation regarding different soil origins but no relationship to BNYVV resistance abilities (4) soil-borne fungi may play an important role with regard to disease severity of BNYVV, but no experimental proof for the presence of soil-borne fungi and a connection to virus resistance-breaking abilities was detected in this study.

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