Occurrence, spread and pathogenicity of different

Beet necrotic yellow vein virus (BNYVV) isolates

Vorkommen, Verbreitung und Pathogenität verschiedener Isolate
des *Beet necrotic yellow vein virus* (BNYVV)

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ABSTRACT

Rhizomania (beet necrotic yellow vein virus, BNYVV) represents an important sugar beet disease, which is transmitted by the biotrophic plasmodiophoromycete *Polymyxa betae*. As long as the disease is not controlled it can lead to yield losses up to 90%. To date yield losses due to BNYVV infestation are inhibited by cultivating resistant sugar beet genotypes, which restrict the virus replication and translocation from infected hair-roots to the taproot. The BNYVV resistance is provided in marketable sugar beet varieties by two major resistance sources (*Rz1* and *Rz2* which either occur singular or in combination). But meanwhile on sugar beet genotypes carrying one (*Rz1*) as well as two resistance genes (*Rz1+Rz2*) resistance breaks could be observed at several BNYVV A-type infected sites in the USA and in Spain. To confirm these observations a 12 weeks greenhouse resistance test with three different cultivars (two partial resistant genotypes containing either *Rz1* or *Rz1+Rz2* resistance sources as well as a susceptible genotype) under standardized conditions with naturally infested soils from 6 locations was performed. The single resistance (*Rz1*) was compromised in soils from Spain (D), France (P-type, RNA-5 containing), and the USA (IV and MN); in reference soils from Italy (R, A-type) and Germany (GG, B-type) *Rz1* resistant sugar beets were not affected. Overcoming of *Rz1+Rz2* resistance after 12 weeks could only be observed in D soil. Over and above the genomic region that encodes for the pathogenicity factor (P25) of the BNYVV RNA3 from beets grown in all soils was analysed.

Previously suggested correlation between "valine" on position 67 of P25 and a higher virulence could not be confirmed. Isolates in one of the soils as well as experiments previously published, where overcoming of resistance could be observed, contain several other aa_{67} than valine. Analyses of additional soil borne pathogens using ITS sequencing and database comparison showed the presence of three pathogens (*Rhizoctonia solani* Keskin, *Fusarium sp., Pythium sp.*). Synergism between BNYVV, *Rhizoctonia solani* Keskin and *Pythium sp.* could lead to severe virus symptoms and weight reductions particularly in the Spanish soil.

To determine if resistance breaks are correlated with the BNYVV inoculum concentration a "Most Probable Number"(MPN) - tests was conducted where same soils as in the resistance tests were examined. Thereby, D soil revealed the highest BNYVV density, the GG soil on the other hand displayed 520 times lower MPN. In order to obtain information on the aggressiveness of particular virus isolates an additional MPN with *Rz1+Rz2* genotypes was performed. Within this test D, IV, MN and P resulted again in high BNYVV densities even able to infect *Rz1+Rz2* plants after 4 weeks cultivation. These results give strong evidence that high inoculum doses are not responsible for the observed resistance breaks. To prove this conclusion another experiment with normalised

inoculum added to sterile soil was carried out. Within this test three time harvests were conducted after 4, 8 and 12 weeks. Obviously, a significant differentiation of virus isolate vs. genotype correlating to tap root weight was only observed after 12 weeks. Consistently, applying adjusted inoculum density, D, IV, MN and P produced the highest virus contents at 12 weeks. Thus, resistance breaks must be connected to high BNYVV pathogenicity and not to inoculum density.

Additional, experiments were conducted to test the influence of viruliferous *P. betae* zoospore concentrations from various origins, carrying different BNYVV-types. But due to uncertainty how many of the zoospores are actually viruliferous, the data resulted in highly different outcomes, not correlating to the results from tests in naturally infected soil.

Moreover, efforts were undertaken to shorten resistance tests and replace them with time saving artificial sugar beet leaf inoculation via co-infiltration of a BNYVV RNA3 encoding P25 infectious cDNA clone and a red fluorescing marker gene (mRFP). Although, the method itself worked very well in young sugar beet leaves, no differences concerning the sugar beet genotype could be detected. The expected variability of fluorescence intensity comparing susceptible and resistant sugar beet cultivars was not given.

CONTENT

APPENDIX

This thesis is based on following manuscripts, which will be referred to by their Roman numerals:

ABBREVIATION

A	alanine
Aa	amino acid
approx.	approximately
A-type	BNYVV isolate displaying a typical RNA composition - A-types are mainly occurring in southern, western and eastern Europe, as well as in the Northern America
BBSV	beet black scorch virus
BMYV	beet mild yellowing virus
BNYVV	beet necrotic yellow vein virus
BSBMV	beet soil-borne mosaic virus
BSBV	beet soil-borne virus
B-type	BNYVV isolate displaying a typical RNA composition - B-types are mainly occurring in central Europe (Germany, Austria, Switzerland)
BVQ	beet virus Q
BYV	beet yellows virus
C48	progenies from a cross between WB41+WB42 and C37
cDNA	copy DNA
cM	centi Morgan
CP	coat protein
DNA	deoxyribonucleic acid
dpi	days post-inoculation
dsRNA	double stranded RNA
E	glutamic acid
ELISA	enzyme linked immunosorbent assay
G	glycine
GST	glutathione-S-transferase
H	histidine
I	isoleucine
ICTV	International Committee on Taxonomy of Viruses
ITS	internal transcribed spacer
J-type	BNYVV isolate containing similar to the French P-type an additional RNA5 and differing from the P-type by the truncation of four amino acids – J-types commonly occur in Asia
kb	kilo base

kDa	kilo Dalton
L	leucine
LOD	likelihood of odds
LSD	least square difference
MP	movement protein
MPN	most probable number
mRNA	messenger RNA
N / n	number of plants / number of repetitions
NC	negative control
NES	nuclear export signal
NLS	nuclear localization signal
nt	nucleotide
ORF	open reading frame
P	protein
PC	positive control
PCR	polymerase chain reaction
pi	plant introductions
PTGS	post-transcriptional gene silencing
P-type	BNYVV isolate displaying a typical RNA composition and an additional fifth RNA - P-types are mainly occurring in a small region in F (Pithiviers), the UK and in KZ
QTL	quantitative trait loci
R	arginine
RdRp	RNA dependent RNA polymerase
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
Rz1	resistance gene against BNYVV from the “Holly” source
Rz2	resistance gene against BNYVV from the WB42 source
Rz3	resistance gene against BNYVV from the WB41 source
siRNA	short interfering RNA
SSCP	single strand confirmation polymorphism
TGB	triple gene block
TPIA	tissue print immunoassay
V	valine
var	variety
WB41	wild beet 41
WB42	wild beet 42
Y	tyrosine

FIGURES

1. GENERAL INTRODUCTION

1.1. Summary

Rhizomania represents also in the future a risk to the world-wide sugar beet production. Although at present the disease can be successfully controlled with natural derived resistances, the virus itself possesses the potential to extremely reduce yield and sugar content. BNYVV can be divided in four major subgroups by means of sequence divergence (A, B, P, and J-type) with different geographic distribution and number of RNA-segments. The disease can be controlled by cultivating partial resistant sugar beet genotypes. The few resistance genes used in practice at present do not prevent the infection with beet necrotic yellow vein virus (BNYVV) and its vector *Polymyxa betae* (a biotrophic plasmodiophoromycete). Thus, the disease spreads to further sugar beet production areas world-wide. By far more important is the fact that inoculum concentration is increasing on several infested sites. Hence, no long-term recovery from BNYVV in the soil can be expected at those locations. A high selection pressure is exerted on BNYVV by the widespread cultivation of genetically-uniform resistant plant material, which could promote the occurrence of resistance-breaking isolates. Above all, this interaction complexity is related to the fact that the naturally occurring resistances used at present do not grant complete immunity against the virus. The resistance in sugar beet only inhibits virus spread from primary infected lateral to tap roots. Further, high viruliferous *P. betae* inoculum in soil, which is able to overcome existing resistances, has already been observed. Concerns about selection of resistance breaking BNYVV isolates are supported by repeated observations of weaknesses regarding yield and sugar content in variety tests of partial resistant Rhizomania varieties. In addition a detailed molecular characterisation of the virus led to the identification of viral pathogenicity factors (P25 and P26) responsible for the development of typical Rhizomania symptoms like small yellow leaf veins, T-shaped tap roots, brownish vascular and the development of lateral root beard growth. P25 and P26 (only occurring in P- and J-isolates) are also known to be responsible for virus movement from infected lateral roots into the tap root. Sequencing P25 genes of several different BNYVV isolates revealed a high variability depending on the geographic origin of the virus. In geographically separated BNYVV infested sites an independent emergence of resistance-breaking isolates, which exhibit a divergent composition of viral pathogenicity factors, were already detected under certain conditions. The J- and P-types that only occurs in a small region around Pithiviers in France, in Kazakhstan, in Japan and on some sites in England is able to cause severe damage on partial resistant sugar beet. Higher virus titres were detected in lateral and tap roots of sugar beet plants cultivated in P-type soil,

compared to sugar beet grown in A and B soil. At present no information on the involvement of the fungal vector, which provides pathogenicity to the virus in the infection cycle as it enables the virus to cause exponentially increasing multiple secondary infections and thus, increases viral primary infection, exists. To understand these high-complex interactions of virus, vector and host, which all have impact on virus pathogenicity, studies to estimate the impact of inoculum concentration and an establishment of artificial infection procedures are urgently required.

High concentrations of viruliferous *P. betae* can overcome resistance. In addition the vector infection is unaffected by the virus resistance. Thus, verification of occurring resistance breaks in partial resistant Rhizomania varieties due to a high variability of pathogenicity genes in different BNYVV-isolates, which developed independently, was needed. Apparently other soil-borne pathogens like *Rhizoctonia solani*, *Pythium* sp., *Heterodae schachtii* and *Fusarium* sp. also influence the severity of the disease. In order to estimate the current and future disease potential and the durability of BNYVV resistance sources used at present correctly several studies of Rhizomania pathogenicity, depending on the genetic composition of virus and parts of the vector, the vector transmission, the inoculum densities of virus and vector and the influence of other soil borne pathogens, were conducted (manuscripts **I** and **II**).

1.2. Disease history

Rhizomania represents one of the economically most important diseases affecting sugar beet production. It is caused by beet necrotic yellow vein virus (BNYVV) (TAMADA & BABA, 1973), belonging to the genus *Benyvirus* (ICTV, 1997) and transmitted by the soil-borne biotrophic plasmodiophoromycete *Polymyxa betae* Keskin (KESKIN, 1964; DESSENS & MEYER, 1996; ADAMS et al., 2001, RUSH, 2003). The original description of the disease took place in Italy in the 50's in the last century (CANOVA, 1959). Ever since the virus spread into numerous sugar beet production areas world-wide (Asia, the USA, South and Central Europe, Scandinavia) (ASHER, 1993; TAMADA, 1999; LENNEFORS et al., 2000; NIELSEN et al., 2001). It can be assumed that the propagation speed of the disease is still increasing. To date, 1.6 millions hectares were examined for the occurrence of the disease within Europe; 1990 15%, 2000 38% and for 2010 56% of the sugar beet production area were predicted to be BNYVV infected (RICHARD-MOLARD & CARIOLLE, 2001).

1.3. The vector of BNYVV: *Polymyxa betae* Keskin

Polymyxa betae Keskin is a biotrophic plasmodiophorid that is hardly influencing plant growth in the field. Greenhouse experiments displayed slight differences in virulence of various virus-free

P. betae isolates where some isolates apparently reduced tap root growth in sugar beet (GERIK & DUFFUS, 1988, BLUNT et al., 1991; KASTIRR et al., 1994).

1.3.1. Vector taxonomy

Polymyxa betae, P. graminis, and *Spongospora subterranea* were considered to be economically unimportant. A proper taxonomy of these soil-borne pathogens was long-term disregarded and led to uncertainness in the last decades when investigations proved the capability of this group to vector viruses. Molecular characterisations proved this group to be unrelated to *Ascomycetes*, *Basidiomycetes*, *Oomycetes*, or *Myxomycetes*, a placement within the *Protozoa* was favoured (BRASELTON, 1995; WARD et al., 2003). Due to characteristics similar to those of plasmodiophorids the recognition as a valid taxonomic group was justified. Typical characters are: obligate intracellular parasitism, cruciform nuclear division, zoospores with two, anterior, unequal whiplash flagella, multinucleate plasmodia, and environmentally resistant, long-living resting spores (cysts) that are often clustered together to form a sporosorus (cystosorus). Development of zoospores and long-living sporosori are also typical for plasmodiophorids (ADAMS, 1990; BARR, 1992; BRASELTON, 1995/2001; LITTELFIELD & WHALLLON, 1999; SHERWOOD & RUSH, 1999).

1.3.2. Life cycle of Polymyxa betae and host range

The life cycle of *P. betae* has been well documented (ADAMS, 1991; BARR & ASHER, 1996; CAMPBELL, 1996; LITTELFIELD & WHALLON, 1999). The protist is able to survive in thick-walled clustered resting secondary spores (sporosori) in the soil for years. There is no indication for virus multiplication in these sporosori (CAMPBELL, 1996). This implies that fields, once infested with viruliferous *P. betae,* neither recover from Rhizomania infestation through the lengthening of crop rotation nor the cultivation of nonhost crops. As soon as a host for *P. betae* is present and a soil displays near-saturated moisture conditions and the temperatures are suitable (ideal for *P. betae* propagation are temperatures around 25°C) the resting spores start to germinate and release virus carrying primary zoospores. Once the primary zoospores contact host cells they encyst immediately and inject the zoospore contents within approx. 2 hours. The primary zoospore develops into a multinucleate plasmodium. Then following two developments are possible either it develops to a sporogenic plasmodium and transforms to a zoosporangium releasing secondary zoospores or it changes into a sporogenic plasmodium converting to a sporosorus to rest in the soil. If secondary zoospores are released, they are actively swimming to new roots cells to infect them. Under favourable conditions (+25°C, high moisture soil at pH 6-8) one cycle can be completed within 60 h (reviewed in ASHER & BLUNT, 1987). The *P. betae* life cycle is schematically demonstrated in Fig. 1.

Fig. 1

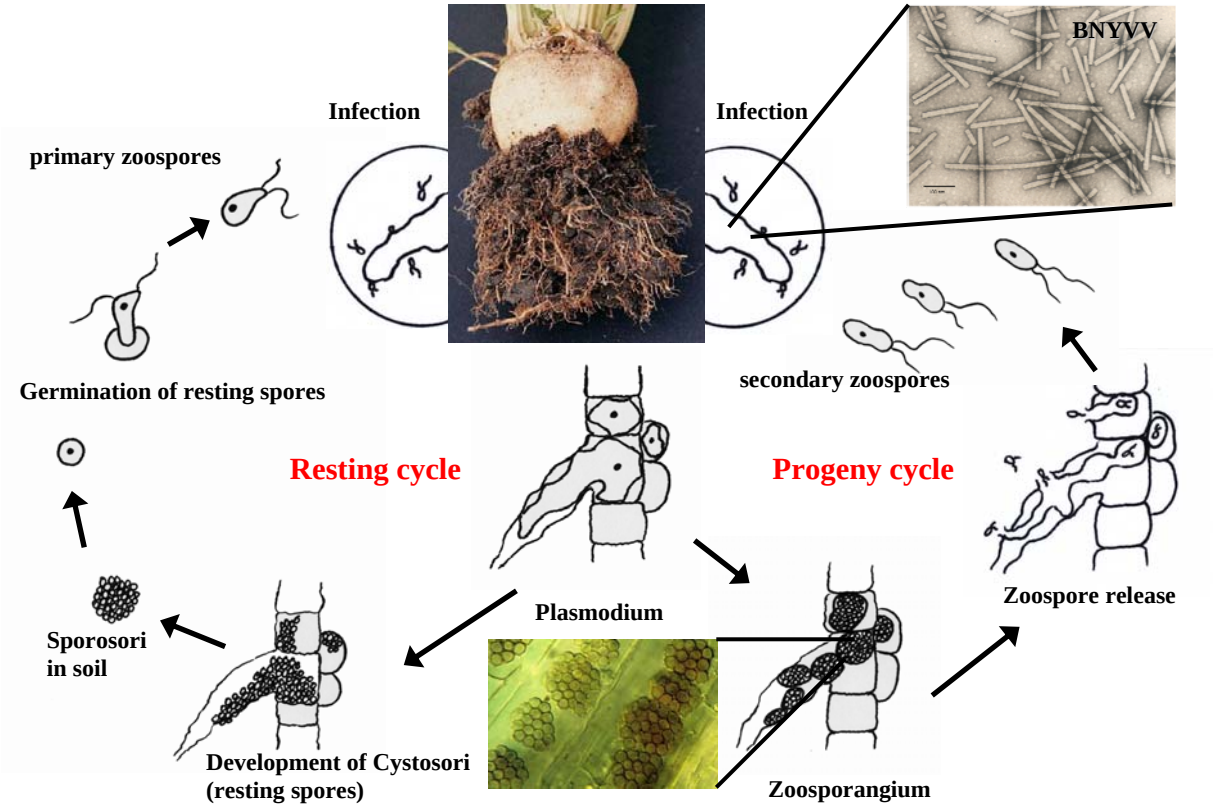

Fig. 1: Life cycle of viruliferous *Polymyxa betae* (mod. after Ruppel, unpublished)

Polymyxa betae and *P. graminis* are morphologically hardly distinguishable, thus both plasmodiophorids were separated by their host range (BARR, 1979; BARR & ASHER, 1992; BRASELTON, 1995). Today, the classification is done after molecular characterisation, strictly separating both plasmodiophorids and eliminating the hypothesis *P. betae* being a forma specialis of *P. graminis* (ADAMS & WARD, 1999; LEGRÉVE et al. 1998, 2000, 2002). *P. betae* possesses a rather small host range that is limited to species within *Chenopodiaceae*, *Amaranthaceae*, *Caryophyllaceae*, and *Portulacaceae. P. graminis* has a much greater host spectrum and is capable to infect both monocotyledonous as well as dicotyledonous species. Most *P. graminis* are able to infected sorghum and millet but vary in their ability to infect wheat, barley, and rye.

1.3.3. Molecular characterization of Polymyxa species

LEGRÉVE et al. (2002) conducted studies on a region of the nuclear ribosomal DNA containing the internal transcribed spacer 1 (ITS1), the 5.8S DNA and the internal transcribed spacer 2 (ITS2) for molecular characterisation of *Polymyxa betae* and *P. graminis*. In theses studies ITS sequences from isolates of *Olpidium brassicae*, *Spongospora subterranea*, *Plasmodiophora brassicae* and *Ligniera* spp. were compared to *Polymyxa graminis* and *P. betae*. However, LEGRÉVE et al. (2002 / 2003) suggested a grouping of *P. graminis* into five different distinct forma specialis: *P. graminis* f.

sp. *temperata*, *P. graminis* f. sp. *tepida*, *P. graminis* f. sp. *tropicalis, P. graminis* f. sp. *subtropicalis*, and *P. graminis* f. sp. *colombiana*. *P. betae* was also included in this sequence comparison. It was strictly separated from *P. graminis*. Further phylogenetic diversification within *P. betae* regarding its origin and the BNYVV-type carrying were not studied. Marginal sequence distinctions within *P. betae* isolates concerning the geographical origin were studied in manuscript **I**.

1.3.4. Vector detection and quantification

Polymyxa spp. are biotrophic organisms so that isolation and artificial cultivation on culture medium is impossible. GERIK (1992) described a selective growing media to grow the soil borne parasite in vitro on selective media in association with root cultures. After inoculating young sugar beet seedlings with *Agrobacterium rhizogenes*, which stimulates proliferation of fine rootlets, these cultures could be established. Because this in vitro method was not very reliable, most scientists use in practice naturally infested soil or infected sterile soil with infested dried root pieces to conduct field and greenhouse experiments (BOAG, 1986; GERIK & DUFFUS, 1988; TUITERT & HOFMEESTER 1992; TUITERT, 1993; TUITERT & BOLLEN, 1993;HARVESON et al., 1996; WISLER et al. 2003).

1.3.5. P. betae detection methods

Bait plant test: A rather simple test to prove the presence of *Polymyxa* spp. in soil by planting host into naturally infested soils. After only 8 days the sporosori can usually be observed on lateral hair roots by microscopy. For good *Polymyxa* spp. propagation the soils should be watered to near-saturation (ABE, 1987; GERIK, 1992). Soils for bait plant test should be used fresh as long-term storage influence the initiation to germinate (SHIRAKO & BRAKKE, 1983; LEGRÉVE et al., 1999).

Most probable number (MPN): Dealing with naturally infested soil the inoculum density of *Polymyxa* spp. is difficult to estimate. One solution is the determination of *P. betae* concentration in soil via MPN (CIAFARDINI, 1991; ADAMS & WELHAM, 1995, TUITERT & HOFMEESTER, 1992; TUITERT & BOLLEN, 1993). This technique has often been used to conduct ecological but also epidemiological studies. Combining the MPN with serological virus tests it is possible to estimate the percentage of viruliferous *Polymyxa* spp. in soils (TUITERT, 1990; CIAFARDINI, 1991). The MPN always delivers relative values that need to be statistically analysed to check the reliability of each test (manuscript **II**).

Serological methods: MUTASA-GOTTGENS et al. (2000) and DELFOSSE et al. (2000) developed serological methods to detect soil-borne pathogens using antiserum. Both enzyme-linked immunosorbent assays (ELISA) are suitable for qualitative and quantitative analyses of infected lateral roots, no matter which stage of fungal life development.

Molecular techniques: In general there are two different ways to detect *Polymyxa* spp. by means of nucleic acid based techniques On the one hand, the successful infestation of *Polymyxa* spp. in planta can be detected qualitatively via Polymerase chain reaction (PCR) (LEGRÉVE et al., 2003), quantitatively by real-time PCR (rtPCR) (LEES et al, 2003) or by means of DNA-probes (MUTASA et al., 1993). *Polymyxa* spp. can also be identified and quantified directly from the soil (CAMPBELL, 1996; WARD et al., 2003). Detecting Polymyxa spp. directly from the soil is difficult due to the inhomogeneous distribution of the soil-borne pathogen inhibiting the choice of a representative sample as only small amounts of soil (<10 g) for nucleic acid extraction are needed (CIAFARDINI, 1991; TUITERT & HOFMEESTER 1992). In fact often only a marginal percentage of zoospores are viruliferous (WORKNEH & RUSH, 2004). With molecular methods it is easily possible to diagnose single development stages of the soil-borne pathogen. GITTON et al. (1999) and MUMFORD et al. (2000) developed the verification and quantification by rtPCR for two viruses in parallel (soil borne wheat mosaic virus and wheat spindle streak mosaic virus as well as potato mop top virus and tobacco rattle virus, respectively) but similar methods for vectored viruses are missing for *P. betae* and BNYVV. A qualitative detection of different pathogens (BNYVV, beet soil borne virus, beet virus Q, and *P. betae*) via multiplex reverse transcriptase PCR (mRT-PCR) is published by MEUNIER et al. (2003b).

1.3.6. Virus-vector relationships

Two different ways of virus transmission via fungal vectors are known (CAMPBELL, 1996), first *in vitro* transmission and secondly *in vivo* transmission. BNYVV exhibits *in vivo* transmission (BARR, 1982; ABE & TAMADA, 1986; ADAMS, 1991; CAMPBELL, 1996). Typical *in vitro* virus transmission is demonstrated between *Olpidium brassicae* and tobacco necrosis virus (TNV), where virus transmission is much more independent from the vector. Once, *O. brassicae* transmits TNV virions, which are only absorbed to the surface of fungal membrane, the virus propagates in the host-cell and further spread is independent from the vector. If the host cell dies, fungal independent TNV virions get released into the soil (CAMPBELL, 1996). *In vivo* transmission is characterised by much more efficient virus spread than *in vitro* transmission. As soon BNYVV infected zoospores are released from resting spores and they contact a susceptible host, they start to inject the virus particles into the plant cell. The virus enters the cytoplasm to complete infestation of the cell with BNYVV and starts its replication and genome expression cycle, including virus

movement to adjacent cells. The opposite way around, if a virus-free zoospore infects a cell, which is already infected by BNYVV, the virus will be incorporated by the developed plasmodium what forms a zoosporangium, whereof secondary zoospores will be viruliferous. If the zoosporangium develops a sporogenic plasmodium it will convert to sporosori to rest in the soil, released zoospores, even after years resting in the soil, will be viruliferous. Recently, VERCHOT LUBICZ et al. (2007) proved by immunofluorescence labelling that BNYVV is accumulating in resting and zoospores of its vector *P. betae*. This would lead to the conclusion that *P. betae* is also a host for BNYVV since the virus lives and propagates inside the vector for more than one life cycle. Still most of the virus vectoring process/transfer is unknown; published data has been reviewed by DESSENS & MEYER (1996), REAVY et al. (1998), TAMADA et al. (1996), DIAO et al. (1999) and ADAMS et al. (2001). Any comparable virus-vector relationship as described for *P. betae* and BNYVV and their host the sugar beet are not puplished, yet.

1.4. BNYVV

In field BNYVV symptoms can be observed as yellow patches that are spread in the same direction of farm machinery movement. Due to ploughing, tillage, sowing and harvesting operations, infested soil is spread not only within the field but also to other sites. Thus the acreage of Rhizomania infested field is continuously increasing (RICHARD MOLARD & CARIOLLE, 2001). On weaker damaged sugar beets, pale, long and upright growing leaves can be observed. Whereas severe symptoms like rudimentary developed tap roots and extreme root beard development consisting of dark brownish lateral roots as well as systemic spread to leaves, causing yellow veins (TAMADA & BABA, 1973; TAMADA, 1975; JOHANSSON, 1985; ASHER, 1993) are nowadays rarely monitored since the cultivation of partially resistant sugar beet in most of the infested sugar beet growing areas. White sugar and root yield can be reduced up to 90% in susceptible cultivars (JOHANSSON, 1985). ASHER (1993) reported the broad possibilities of Rhizomania to spread into none or less infested field via seed potatoes and onions, farm machinery, irrigation, flooding and wind erosion.

1.4.1. Virus taxonomy

Since 1997 BNYVV is accepted by the International Committee on Taxonomy of Viruses (ICTV) as member of the genus *Benyvirus*. BNYVV consists of 4-5 rod-shaped particles, which encapsidate 4-5 genomic ss (+) strand RNAs, depending on the isolates (BOUZOUBAA et al., 1985, 1986, 1987; TAMADA et al. 1989; KIGUCHI et al., 1996; KOENIG et al. 1997).

1.4.2. Genome organisation of BNYVV

The BNYVV genome organisation and the known functions of the viral gene products expressed is displayed in Fig. 2.

Fig. 2

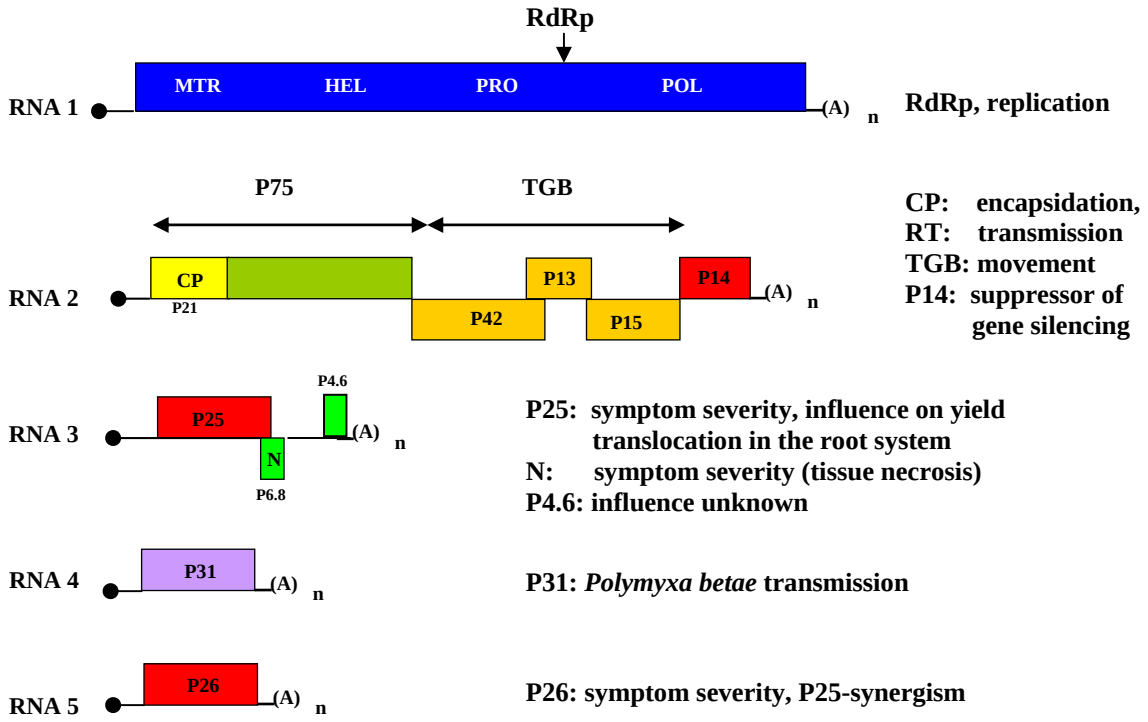

Fig. 2: Beet necrotic yellow-vein virus (BNYVV) genome expression und translation strategy, subdivided in five RNA segments, whereas only P- and J- types obtain the fifth RNA. All segments possess a cap structure at the 5´end and a poly A-tail (A) at the 3´end. Each box displays an open reading frame (ORF) in the genome, colours indicate the gene functions (blue = replication, yellow = coat protein, green = vector interaction, orange = cell-to-cell movement, red = pathogenicity, light green = connected to pathogenicity, but further functions are still unknown, lilac = cell-to-cell movement (vector transmission). RdRp = RNA dependent RNA polymerase, CP = coat protein, RT = readthrough protein, TGB = triple gene block, N = ORF inducing tissue necrosis only when sequences upstream are deleted.

RNA1

The RNA1 (in total 6746 nucleotides long, excluding the poly(A)-tail) encodes an ORF for a 237 kDa polypeptide possessing motifs for methyl transferase, helicase and RNA dependent RNA polymerase (RdRp). Thus the RNA1 is assumed to function as viral replicase protein (BOUZOUBAA et al., 1987). By a papain-like protease activity between the helicase and RNA dependent RNA polymerase (RdRp) motifs this protein is processed into a 150-kDa and 66-kDa product (the latter containing the polymerase domain) (HEHN et al., 1997).

RNA2

On the RNA2, 4612 nucleotide in length [excl. the poly(A)-tail] are genes located for fundamental viral functions such as replication, cell-to-cell movement, encapsidation and suppression of post-transcriptional gene silencing (PTGS) (TAMADA, 1999; DUNOYER et al., 2002). RICHARDS et al. (1985) and ZIEGLER et al. (1985) have demonstrated that the M_r 22 000 (P22) viral coat protein (CP) is encoded by RNA2. Together with an 85 kDa polypeptide both proteins are immunoprecipitated by antiserum against BNYVV (e.g. in ELISA). In total the RNA2 possess 6 open reading frames (ORF). The first 5´ORF codes for a 21 kDa (P21) CP with a rather weak UAG termination codon (RICHARDS et al., 1985; ZIEGLER et al., 1985). A 75 kDa protein following P21 is a read-through (RT) protein that is involved in virus assembly and vector transmission (ZIEGLER et al., 1985). The next three 3´-located ORFs represent the triple gene block (TGB) encoding 3 movement proteins (MP) and perform in a highly specific manner (LAUBER et al., 1998). The P42 is able to bind single and double stranded RNA and DNA and thus it can bind the viral genomic RNA (BLEYKASTEN et al., 1996), P13 and P15 both may be able to connect to the plasmodesmata to allow the P42 to enter and modify the plasmodesmata to enable BNYVV-particle cell-to-cell movement (NIESBACH-KLÖSGEN et al., 1990, LAUBER et al., 1998; ERHARDT et al., 2000). GILMER et al. (1992) described the last ORF, a 14 kDa (P14) protein, which obtains regulatory functions. P14 is known to be able to suppress a natural virus defence mechanism of plants called RNA silencing (DUNOYER et al., 2002).

RNA3

The RNA3 [1175 nucleotide in length excluding the poly(A)-tail containing in total 3 ORFs] is responsible for symptom development in sugar beet roots and the formation of local lesions in experimental hosts like *Chenopodium quinoa* (TAMADA et al., 1989; COMMANDEUR et al., 1991; KOENIG et al., 1991; JUPIN et al., 1992). Especially P25 (a 25 kDa protein, nucleotide position 445-1102) is considered to be the most important functional protein on RNA3 regarding BNYVV symptom severity in roots.

An efficient BNYVV translocation in the root system of susceptible sugar beet genotypes is only possible if an intact P25 is present (KOENIG & BURGERMEISTER, 1989). TAMADA et al. (1989) proved via ELISA that partial resistant sugar beet seedlings impede the BNYVV spread from primary infected lateral roots to the tap root. However, CHIBA et al. (2003) inoculated different susceptible and partial resistant sugar beet leaves and observed on partial resistant cultivars only with the presence of RNA3 the formation of necrotic lesions (hypersensitive resistance, HR) or the absence of lesions (infection resistance). Hereupon, the authors concluded a close correlation

between leaf-reactions and resistance abilities in roots of sugar beet plants. These investigations are supporting the hypothesis that BNYVV P25 acts in resistant genotypes as avirulence gene product (*avr* gene) and in susceptible genotypes it represents the BNYVV pathogenicity factor. Thus, the authors concluded that P25 could be responsible for systemic infections in susceptible sugar beet genotypes. P25 is a highly variable protein that mainly diversifies between a specific amino acid (aa) tetrad position 67-70 (SCHIRMER et al. 2005). Additional high variability could be observed on aa position 135 (RUSH et al., 2006). First predicted correlations between specific tetrad compositions and the pathogenicity of BNYVV could not be evidenced (LIU & LEWELLEN, 2007).

HEABERLÉ & STUSSI-GARAUD (1995) demonstrated the presence of P25 in cytoplasm and nuclei of infected cells via immuno-gold electron microscopy. VETTER et al. (2004) examined P25-functions depending on its subcellular localisation in *C. quinoa*. By confocal laser scanning microscopy analysis of wild-type and mutated P25 fused to green fluorescent protein (GFP) a nuclear localization signal (NLS) in the N-terminal part of the protein could be identified. Hereupon, a nuclear export signal (NES) was characterized by mutagenesis. The development of necrotic lesions depends on the subcellular localization of P25. Studies revealed that accession of P25 into both, the cytoplasm and nuclear compartments, led to enhanced symptom severity in *C. quinoa* (VETTER et al., 2004).

The influence on symptom severity of additional ORFs (N and 4.6) (BOUZOUBAA et al., 1985, JUPIN et al., 1991) are still unknown. ORF N may result in strong necrotic symptoms on leaves when overlapping sequences of 3´terminal P25 are deleted. JUPIN et al. (1992) also stated that ORF N could induce leave and root symptoms under natural infestation of BNYVV. Moreover P4.6 did not show any influence on symptom severity in the same publication.

RNA4

The BNYVV RNA4 is a rather small RNA segment (1431 nucleotides) and encodes the P31 protein (BOUZOUBAA et al., 1985). P31 is necessary for vector transmission by *P. betae* (TAMADA & ABE, 1989). RAHIM et al. (2007) describes that P31 posesses next to vector transmission also suppressor of gene silencing function. The role of an additional ORF containing a 6.5 kDa protein is unknown, yet (JUPIN et al., 1991).

RNA5

The RNA5 occurs only seldom in Western Europe (France and the UK) it is more common in Japan (MIYANISHI et al., 1999). Both in France and in the UK, BNYVV isolates containing a fifth

RNA are spread on limited geographic regions (KOENIG et al., 1997; HEIJBROEK et al., 1999; KOENIG & LENNEFORS, 2000; HARJU & RICHARD-MOLARD, 2002, WARD ET AL., 2007). RNA5 encodes protein of 26 kDa in size (P26), likely to act in synergistic manner with P25 thus involved in symptom development and symptom severity, too (KIGUCHI et al., 1996; TAMADA et al., 1996; KOENIG et al., 1997; MIYANISHI et al., 1999). Sequence homology between P25 (RNA3) and P26 (RNA5) as well as a highly conserved aa motif support the hypothesis to deal with an additional pathogenicity factor in BNYVV-P-isolates, responsible for resistance breaking abilities of RNA5 containing strains (KOENIG et al., 1997). SCHIRMER et al. (2005) proved high aa variability at positions 9, 81 and 143 in P26. If variable pathogenicity between single BNYVV RNA5 containing isolates exist has not been experimentally proven, yet. TAMADA et al. (1996) reported about synergistic effects of RNA3 and RNA5 that result in stronger symptom severity compared to BNYVV isolates that only contain RNA1 to 4 (KOENIG et al., 1997b; HEIJBROEK et al., 1999; LINK et al., 2005). It can be assumed that this synergism is confined to P25 and P26.

P26 was also examined concerning the subcellular localization and its pathogenicity functions in *C. quinoa* were characterized in detail (LINK et al., 2005). The authors could prove the localisation of P26 in the cytoplasm and nuclear compartments of infected cells, similar to P25. Beyond that, transcriptional activation and the involvement of P26 in BNYVV formation of local lesions in *C. quinoa* were demonstrated.

1.4.3. BNYVV variability

Over the last decades the existence of different genotypic BNYVV groups became obvious, they correspond to differences in pathogenicity and specific geographical regions (KOENIG et al. 1995; KOENIG et al., 1997; KOENIG & LENNEFORS, 2000; MEUNIER et al., 2003a; TAMADA et al., 2003) as demonstrated in Fig. 3.

Fig. 2

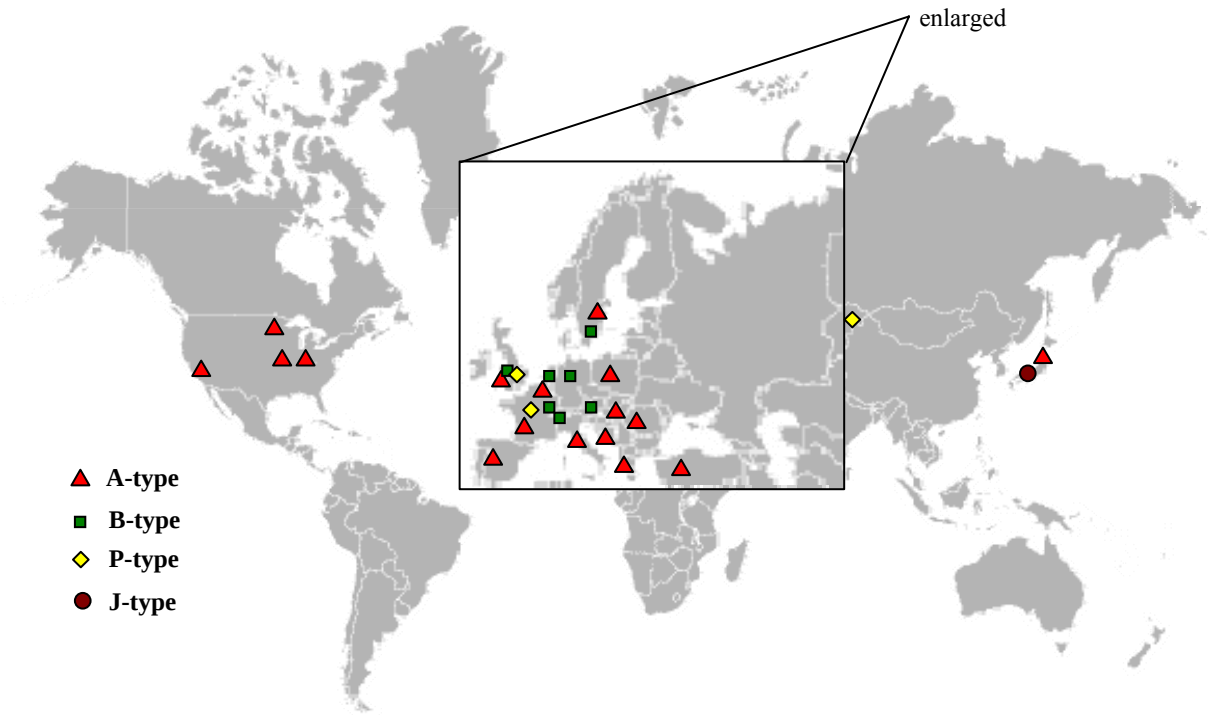

Fig. 3: Distribution of different BNYVV-types depending on the geographic origin

In Europe three different strains of BNYVV were characterized as A-, B- and P-types. Firstly, the separation of strains was based on restriction fragment length polymorphism (RFLP) analysis or single-strand conformation polymorphisms (SSCP) analysis of the CP region from BNYVV isolates originating from all over the world (KOENIG et al., 1995; KRUSE et al., 1994). The A-type is widespread in Europe, the United States, Japan and China. The B-type is less prevalent; it is mainly concentrated on fields in Germany, France and in Switzerland. Differences in nucleotide sequence between A and B range from 3 to 6 %, depending on the genomic RNA analysed. Sequences within A- and B-types are highly conserved and very stable what is also displayed in ≥99% sequence identity (KOENIG & LENNEFORS, 2000). Nucleotide differences cannot be distinguished serological, since aa changes in the viral CP between A and B strains are located in regions that are unrecognized by BNYVV antibodies. SSCP analyses or detection of the additional RNA5 / P26 via PCR are used to distinguish BNYVV-P from -A and -B (KOENIG et al., 1997). Recently, SCHIRMER et al. (2005) conducted analyses of the nucleotide sequences of the RNA2-encoded CP, RNA3-encoded P25 and RNA5-encoded P26 proteins. Phylogenetic trees showed a clear classification of various BNYVV-isolates into different groups which are closely correlated between the virus clusters and geographic origins. Whereas the CP showed to be most conserved in all BNYVV isolates, P26 was less restrained and P25 had by far the highest variability in its nucleotide composition. Especially the tetrad composition 67-70 (as above mentioned) possesses highly variable nucleotides depending on the BNYVV isolate.

Several studies have been conducted to investigate the increased virulence of BNYVV isolates containing RNA5. MIYANISHI et al. (1999) and TAMADA et al. (2003) accomplished experiments to characterize if variability is associated to virulence or geographical distribution. MIYANISHI et al. (1999) established three groups of RNA5 containing isolates after sequence comparison of P26. Most isolates from China and Japan were belonging to group I, two further isolates from Japan were summarized as group II and the French isolate pertain to group III. Differences in the genome within one group were only marginal (approx. 0.6%), between groups sequences varied up to 8%. SCHIRMER et al. (2005) distinguished the Japanese RNA5 groups and the French RNA5 isolates from Pithiviers due to the high sequence variability into BNYVV-P and BNYVV-J isolates.

The genetic variability of RNA plant viruses represents a main factor of virus pathogenicity. Undirected mutations as genetic adaptation take place constantly due to changing environmental condition. Nonviable mutants develop continuously; in addition, emergence of variability retains the fitness of the pathogens (DRAKE & HOLLAND, 1999; GARCIA-ARENAL et al., 2003). The extensive and exclusive cultivation of hosts with only few resistance sources inhibit the virus to replicate and efficiently to produce severe symptoms on sugar beet plants. But as in partial resistance sugar beets the virus can replicate at least in small rates, the selection of isolates overcoming resistance can be more probable than in plants possessing BNYVV immunity.

1.5. Virus-host interaction

1.5.1. Factors influencing disease spread and severity

In field, Rhizomania starts with appearance of single sugar beets that display fluorescent yellow leaves in the mid-growing season. In fields where no BNYVV was observed previously these single “Rhizomania-spots” usually appear due to dumped infested soil from farm machinery, tare soil or after flooding events near rivers etc. Once viruliferous *P. betae* was introduced in a non-infested field via infested soil or sugar beet residues, the protist exhibits an extremely high multiplication potential. Under suitable conditions *P. betae* possesses the capability to multiply more than 10.000-fold within one growing season (TUITERT & HOFMEESTER, 1992). Studies revealed that high BNYVV densities in soil (high concentrations of viruliferous *P. betae*) are closely related to symptom severity (TUITERT, 1990; CIAFARDINI, 1991; TUITERT & HOFMEESTER, 1992; TUITERT et al., 1994). Regarding a whole field, the inoculum density is often determined to be rather low, only in local spot it results in tremendous densities due to the fact, that *P. betae* is in general not widespread (RUSH & HEIDEL, 1995). As soon a field with few diseased spots is machined (tillage, ploughing, sowing operations), soil including viruliferous vector is spread further on and the multiplication of *P. betae* extraordinary increases again once a host is cultivated.

Rhizomania spread in field via zoospores migration is negligible compared to tillage, sowing, irrigation and harvest operations (HARVESON et al., 1996). TUITERT (1993) demonstrated that viruliferous *P. betae* zoospores are not able to bridge a distance of 5 cm between infected and virus-free sugar beet roots. Thus, a high density of resting sporosori which are capable to spread by soil movement is more sufficient to distribute Rhizomania in field than the plant-to-plant infestation via secondary zoospores.

The performance of viruliferous *P. betae* in soil is influenced by many different biotic and abiotic factors. Soil temperature, moisture and structure play important roles in the infection and virus transmitting process. ABE (1987) and BLUNT et al. (1991) reported about an optimal temperature between +25°C and +30°C. Temperatures of +10°C to +15°C inhibit the infection with the BNYVV vector. Especially, sandy soils enforce the *P. betae* zoospore release due to quicker warming (WEBB et al., 2000). While sowing and in young seedling age, seldom temperatures rise to temperatures above 15°C in sugar beet production areas, thus infestation in early stages are uncommon in field. But greenhouse experiments showed that higher temperatures resulted in strong infestation of BNYVV and in maximum symptom severity and weight loss of sugar beet seedlings cultivated in soil with viruliferous *P. betae* (manuscript **II**) depending on *P. betae* population. In the field this incidence may occur if sugar beets are replanted later in the vegetation period, cultivated as winter crop (sowing in August / September) or if the spring is extremely hot compared to average years. Soil moisture is one of the most important factors to add up to successful *P. betae* zoospore release, the initiation of sufficient host infestation. Soil moisture near-water saturation is indispensable to stimulate germination of sporosori (HARVESON & RUSH, 1993; PICCINNI & RUSH, 2000). TUITERT & HOFMEESTER (1992) reported about a significant higher BNYVV infestation of sugar beet cultivars in irrigated than not irrigated soils. Thus, the more viruliferous zoospores are released due to cultivation of susceptible cultivars or due to overcoming of resistance, the higher is the inoculum density, symptom severity and proximate yield loss (RUSH, 2003).

Biotic factors that influence disease incidence are manifold. Not only inoculum density but also *P. betae* origin (population), presence of aviruliferous *P. betae* and the availability of hosts susceptible to *P. betae* and BNYVV are fundamental factors for successful infestation. GERIK & DUFFUS (1988) described differences in vectoring abilities of *P. betae* populations depending on their origin. They also reported that viruliferous vector multiplied much more efficient when an indigenous avirulent population was already present in soil, compared to soil where no *P. betae* population is existent, whereas a virulent population is out-competing aviruliferous *P. betae*. In contrast, KASTIRR et al. (1994) reported that viruliferous vectors are less aggressive and the final Rhizomania inoculum density in soils and sugar beet roots is decreasing. TUITERT & HOFMEESTER

(1992) demonstrated that viruliferous *P. betae* are capable to extremely increase its density within one growing season when an avirulent population already exists; still the percentage of viruliferous zoospores were estimated on only 5% to 20% (CIAFARDINI, 1991; TUITERT et al. 1994). The host plant susceptibility to BNYVV has an immense impact on the development of virus inoculum densities. As soon susceptible hosts are cultivated the inoculum concentration increased quickly and as long resistant sugar beets are cultivated the inoculum density was estimated to be lower (ABE & UI, 1986; ABE, 1987; TUITERT et al. 1994; BÜTTNER et al., 1995; HUGO et al., 1996). Unless new virulent strains of BNYVV are developed, or the primary inoculum in soil is that high that even resistance can break. Experiments have shown that resistance against BNYVV does not apply for *P. betae*.

1.5.2. Genetic resistance against Rhizomania

First resistance breeding programs to select Rhizomania partial resistant sugar beets within variety tests started already in the late 1970s. Reduced or missing virus symptoms, increased sugar beet and white sugar yield as well as processing quality served as selection criteria (FUJISAWA et al., 1982; BÜRCKY, 1987). Genotypes that were chosen for further breeding processes displayed like susceptible sugar beet a high BNYVV content, still they suffer the virus infestation in lateral roots but showing little less symptoms and better field performance (BÜRCKY, 1987). Thenceforward, sugar beets that tolerated the virus infection in lateral roots but accomplished better yield were described as partial resistant sugar beets. JOHANNSON (1985) described a correlation of BNYVV partial resistant sugar beets to resistance of *Cercospora beticola.* Onward, sugar beet varieties like "Dora" and "Lena" (BOLZ & KOCH, 1983; HECHT, 1989) were merchandised as partial resistant cultivars. The first variety showing higher resistance against the disease was the cultivar "Rizor" that was developed from Italian germplasm (DE BIAGGI, 1987). After GIUNCHEDI et al. (1985 & 1987) published a correlation between virus concentration in sugar beet tap roots and white sugar yield the BNYVV content was used as suitable selection criteria in resistance breeding processes. Time and labour intensive selection for partial resistant sugar beets could even be shortened as sugar beet seedlings distinguish already after four weeks in virus content of lateral roots concerning the resistance abilities (BÜRCKY & BÜTTNER, 1985; BÜTTNER & BÜRCKY, 1990). This criterion was comprised when testing one of the most important Rhizomania resistance sources. The so called "Holly" resistance was first identified in 1983 in a sugar beet field trial conducted by the Holly Sugar Company in California, USA (LEWELLEN et al., 1987). The "Holly" source contains the partially dominant resistance gene named *Rz1* as well as further minor genes which have not been identified yet (LEWELLEN et al. 1987; SCHOLTEN et al., 1996; PELSY & MERDINOGLU, 1996). *Rz1* is today's most important BNYVV resistance gene (BIANCARDI et al. 2002). However, *Rz1* is not performing equally in all genetical backgrounds or hybrid cultivars (RUSH et al., 2006).

Occurrence of lower resistance stability and severity under high inoculum densities of diploid compared to triploid hybrid cultivars can be explained by the additive effects of minor genes (BIANCARDI et al., 2002). Marker assisted selections make control and succession of *Rz1* in breeding programs easy and accelerate breeding progress in backcrossing and population improvement programs (FRANCIS et al., 1998; PELSY & MERDINOGLU, 1996; SCHOLTEN et al. 1997). Although breakthrough marketable partial resistant sugar beet genotypes have been developed, including *Rz1*, sugar beets are still BNYVV hosts and the virus is able to replicate in lateral hair roots. Thus, Rhizomania inoculum is continuously increasing in soil. SCHOLTEN et al. (1994) described that resistance is depending on inoculum density in soil, high BNYVV concentrations are overcoming *Rz1* resistance after *in vitro* inoculation with high numbers of viruliferous *P. betae* zoospores.

Since single dominant resistance genes (*Rz1*) loose resistance abilities due to selection pressure they exert on the pathogen population (reviewed in RUSH et al., 2006) the search of additional natural occurring resistance genes was indispensable. The search was expanded to additional germplasms from *Beta vulgaris* susp. *maritima* e.g. from Denmark. Within germplasm specific individual accessions (plant introductions, pi) were backcrossed into sugar beet lines, thereinafter it was identified in greenhouse (WHITNEY, 1989) and in field (LEWELLEN, 1995; LEWELLEN & WRONA, 1997). The resistance was generated from the wild beet (WB) WB42 and inbred as described above thereinafter it was released as accession C48 (LEWELLEN & WHITNEY, 1993). Since the resistance source displayed a higher degree of resistance towards Rhizomania (WHITNEY, 1989) and SCHOLTEN et al. (1994 & 1999) could show that this resistance was located at a different locus on chromosome 3 only few centiMorgan (cM) apart from *Rz1* it was named *Rz2*. Most other resistance sources published are either *Rz1* or *Rz2*. In mass selection individually screened resistant plants were pooled and different populations were developed wherein the actual resistance source of each population was unknown (DONEY et al., 1990). Recently, GIDNER et al. (2005) identified an additional major resistance gene (*Rz3*) in WB41. *Rz3* is also mapped on chromosome 3 of the sugar beet genome separately from *Rz2*. The influence on resistance performance of other minor genes next to *Rz3* cannot be excluded, too. An association of different quantitative trait loci (QTL) with BNYVV resistance independent from *Rz* resistance genes on linkage group 3 has been observed (KRAFT, pers. comm.). GIDNER et al. (2005) demonstrated lower BNYVV contents in partial resistant sugar beets possessing a combination of *Rz1* and *Rz3* in a heterozygous condition, than in plants containing only *Rz1*.

Since the multiplication rate of BNYVV in partial resistant sugar beets is much lower than in susceptible cultivars, ASHER & KERR (1996) forecasted a stabilisation of inoculum density in soil. The cultivation of partial resistant Rhizomania varieties is increasing continuously (in Germany

2007 almost 70% of sugar beets grown possessed at least one resistance gene (LADEWIG pers. comm.). This would include a reduction of the velocity of propagation in soil but experimental evidence is missing.

A further possibility to achieve BNYVV resistance can be realised by generation of transgenic virus resistant plants (LENNEFORS, 2007). Previously, coat protein mediated resistance was generated by using translatable coat protein genes to transform the sugar beet plant. This mechanism is based on the disruption of functions in viral multiplication and provided good Rhizomania resistance in field and greenhouse (MANGOLD et al., 1998; MECHELKE & KRAUS, 1998; SCHOLTEN & LANGE 2000). Nontranslatable genes and gene-fragments are also used to generate virus resistance in sugar beets. By expressing double stranded RNA (dsRNA) or specific RNA concentration, an internal resistance mechanism *in planta* named "RNA silencing" is activated (BAULCOMBE, 2004 / 2005; FILIPOWICZ et al., 2005). "RNA silencing" is initiated by dsRNA, it causes sequence specific degradation of virus RNA what acts as adaptive resistance mechanism. The temporary production of dsRNA, which induces this resistance mechanism as soon the virus multiplies in planta, functions almost in every plant. That "RNA silencing" is not always successfully inhibiting virus spread is depending on the virus. The virus is able to suppress "RNA silencing" by evolving proteins which interfere with the resistance mechanism (SILHAVY & BURGYAN, 2004; VOINNET 2005). By transgenic expression or induction of viral dsRNA genetically transformed plants are able to initiate the resistance mechanism against viruses before the actual virus infection takes place (WATERHOUSE et al., 1998; SMITH et al., 2000; CHEN et al., 2004; HELLIWELL & WATERHOUSE, 2005). To generate this kind of virus resistance only fragments of viral genes are expressed what is promising and much more sustainable, since discussed biological risk of transgenic virus-resistant plants due to recombination and complementation are minimised (AAZIZ & TEPFER, 1999). However, at present transgenic BNYVV-resistant sugar beet based on the "RNA silencing" mechanism are tested (KRAUS, pers. comm.). Concerning the acceptance and political situation towards genetically modified organisms - besides the USA - genetic resistance from naturally occurring resistance sources via classical sugar beet breeding and selection seems to be the favoured way to control the disease.

1.5.3. Other soil-borne pathogens

In naturally BNYVV infested soils additional soil-borne pathogens always occur, which also have sugar beet as host. It can be assumed that additional infections (primary or secondary) are affecting the BNYVV content, also in partial resistant sugar beets (STEVENS & ASHER, 2005). The authors have shown in field trials, that co-infection with beet mild yellowing virus (BMYV) led to increased Rhizomania symptoms even in partial resistant plants. In contrary LENNEFORS (2007)

reported about BNYVV co-infection with beet yellows virus (BYV) which did not lead to significant influence on the BNYVV content in lateral beet roots. But BYV displayed higher multiplication rates when secondary viruses are present what can be explained due to competition in the phloem (SMITH, 1991; SMITH & KARASEV, 1991). Combining BMYV and BYV with following soil-borne viruses BNYVV, beet soil borne virus (BSBV) and beet virus Q (BVQ) no interaction or increasing virus titres could be detected (LENNEFORS, 2007). Already in the 1980s CUI (1988) reported about high yield reductions and severe beet black scorch virus (BBSV) symptoms on leaves and tap roots in China. Further spread to other western countries could not be observed since then. But recently, WEILAND et al. (2007) reported about the occurrence of BBSV in the USA that displayed severe symptoms and influenced sugar beet growth similar to Rhizomania. Investigations if BBSV is involved in the intensifying Rhizomania disease in the USA are necessary.

Fungal soil-borne pathogens like *Rhizoctonia solani* Keskin, *Aphanomyces* ssp., *Fusarium* ssp. and *Pythium* ssp. are often occurring parallel to BNYVV (manuscript **I**). An influence of co-infection with these fungi can be assumed. *Rhizoctonia solani* Keskin, *Aphanomyces* ssp. and *Pythium* ssp. are known to infect the sugar beet seedlings in very early stages (5 days after inoculation) (LUTERBACHER et al., 2005) thus it seems plausible that the fungus could set primary infection and BNYVV secondarily damages the plant due to previous impairment, although it is BNYVV resistance. But up to date, no evidence for synergisms between BNYVV and other fungal soil borne pathogens regarding symptom severity could be shown.

1.6. Rhizomania-resistance tests in practice

In general two different kinds of Rhizomania-resistance tests are published. On the one hand there are field trails (often conducted as variety test for commercial seed or seed where registrations are applied). On the other hand Rhizomania-resistant tests are often conducted in greenhouse, either with naturally infested soil or via artificial inoculation.

Former resistance test were conducted always in field which were known to be infested with Rhizomania. The sugar beet plants were scored for yellowing leaves and veins, for crinkling, root rot, brownish vascular and root beards at harvest date after a whole vegetation period (approx. 7 month) (FUJISAWA et al. 1982). Little later GUINCHEDI et al. (1985, 1987) reported about negatively significant correlations between sugar beet yield and virus concentration in the tap root of fully developed sugar beets, a criterion that was also used to describe quality of resistance in susceptible and partial resistant sugar beets. BÜRCKY (1987) proved, that BNYVV infestation not only decreased sugar beet yield but also increased the sodium and potassium contents. These

concentrations were also used to evaluate the severity of Rhizomania infection. After BÜRCKY & BÜTTNER (1985) demonstrated that virus content vs. sugar beet genotypes distinguishes in seedling state, the development of time saving greenhouse tests with standardised climate conditions was encouraged. First resistance test with individually planted sugar beets in naturally infested soil were conducted for six weeks in greenhouse. The virus could only be detected in the lateral roots, not in sugar beet tap roots, anyhow the different genotypes examined distinguished significantly in their virus content. In field tests, however, the virus content in tap roots stayed first choice, because of decreasing virus concentrations in lateral roots over the vegetation period (BÜRCKY & BÜTTNER, 1991).

A couple of greenhouse resistance tests have been published over the years, ABE & TAMADA (1987) developed a greenhouse experiment in which undefined amounts of resting spores and zoospores were added to Rhizomania free soil to infect sugar beet seedlings. Also independent from inoculum concentration trials were conducted by BÜRCKY & BÜTTNER (1985) and PAUL et al. (1992), who used a mixture of naturally infested soil and sand in equal parts and cultivated seedlings for six and four weeks, respectively. Most resistance tests conducted nowadays are following PAUL et al. (1992a) concerning temperature (22/17°C, day/night) and vegetation period (4 weeks). PAUL et al. (1993c) discovered also a correlation between virus content in sugar beet rootlets with sodium and α-amino nitrogen concentration in tap roots of infected sugar beets grown in field. To test for general level of infestation in field / naturally infested soil PAUL et al. (1993c) recommend a bait plant test with a susceptible cultivar. Whereas the number of individually infected susceptible sugar beets serves as criterion to estimate the actual inoculum density in soil using the Most Probable Number (MPN) method (TUITERT, 1990; CIAFARDINI, 1991). Resistance tests with specific inoculum densities were conducted by HEIJBROEK et al. (1999) and SCHOLTEN et al. (1994) in defined amounts of naturally infested soil containing different BNYVV-types or in hydroponics using a defined number of *P. betae* zoospores, respectively. Via inoculum suspensions higher BNYVV inoculum concentrations than in naturally infested soil could be established since overcoming of *Rz1* resistance could be observed (PETERS & GODFREY-VELTMAN, 1989; PAUL et al., 1993b; SCHOLTEN et al., 1994). KOENIG & STEIN (1990) developed a method to artificially inoculate sugar beet seedlings by vortexing without the vector *P. betae* after passaging the virus on *Chenopodium quinoa* leaves. Resistance tests based on mechanical leaf inoculation of sugar beets resulted in very low sensitivity thus they are nowadays uncommon to use (FUJISAWA & SUGIMOTO, 1979; GRASSI et al.; 1988). Up to date, 5-6 weeks resistance test in naturally infested soil prepared as described by PAUL et al. (1993c) but with high numbers of sugar beet seedling per pot (100 sugar beet seeds on 280 ml soil) are widespread to test resistance abilities (LIU at et al., 2005; LIU & LEWELLEN, 2007). Additional attempts to conduct resistance tests following PAUL et al. (1993b) with dried rootlets from infested sugar beets in sterile sand were modified. The analyses for virus

presence were accomplished through ELISA (CLARK & ADAMS, 1977) or tissue print amino assay (TPIA) (KAUFMANN et al. 1992).

2. AIMS OF THE STUDY

The cultivation of BNYVV resistant varieties in Rhizomania infested fields is indispensable to conduct profitable sugar beet production. But, in few sites in the USA and in Spain the cultivation of resistant sugar beet cultivars containing the present available resistance genes like *Rz1, Rz2* or a combination of *Rz1+Rz2* are not withstanding the virus infection and display severe symptoms and high yield losses.

The aim of this study was to evaluate the reason for resistance breaks in partial resistant sugar beet cultivar under specific consideration of virus and vector inoculum density, to analyse the role of the BNYVV vector *Polymyxa betae*, to examine the influence of an extremely variable pathogenicity factor (P25) encoding on the virus RNA3 and to display the temporal development of virus infestation after artificial inoculation.

3. RESULTS AND DISCUSSION

After overcoming resistance (*Rz1*) has been reported in several sugar beet production areas (USA, Spain and France) in greenhouse and field experiments a resistance test including these soils from the USA (IV and MN), Spain (D) and France (P) as well as less aggressive standard soils from Italy (R) and Germany (GG) under standardized greenhouse conditions was conducted in order to clarify the performance of *Rz1* as well as *Rz1+Rz2* sugar beet cultivars. As control a BNYVV susceptible genotype was also included. The sugar beet was analysed after 12 weeks of greenhouse cultivation for tap root weight, virus concentration and relative Polymyxa glutathione-S-transferase (GST) content. The amino acid composition of the pathogenicity factor P25 at position 67-70 (aa_{67-70}) was determined and additional soil-borne pathogens naturally occurring in these soils were identified.

3.1. Cultivation-time-dependent resistance tests

In contrast to commonly accomplished greenhouse resistance tests of four to six weeks following PAUL et al. (1992) with slight modifications (TUITERT et al., 1994; BÜTTNER et al., 1995; SCHOLTEN et al. 1996; HEIJBROEK et al., 1999; LIU et al., 2004; LUTERBACHER et al., 2005; LENNEFORS, 2007) a 12 weeks resistance test was conducted. The cultivation time was extended due to previously performed resistance tests. In this previous test a strong negative correlation of BNYVV content in lateral roots and tap root weight was found after artificial inoculation following a 12 week greenhouse cultivation period (Fig. 4).

Fig. 4

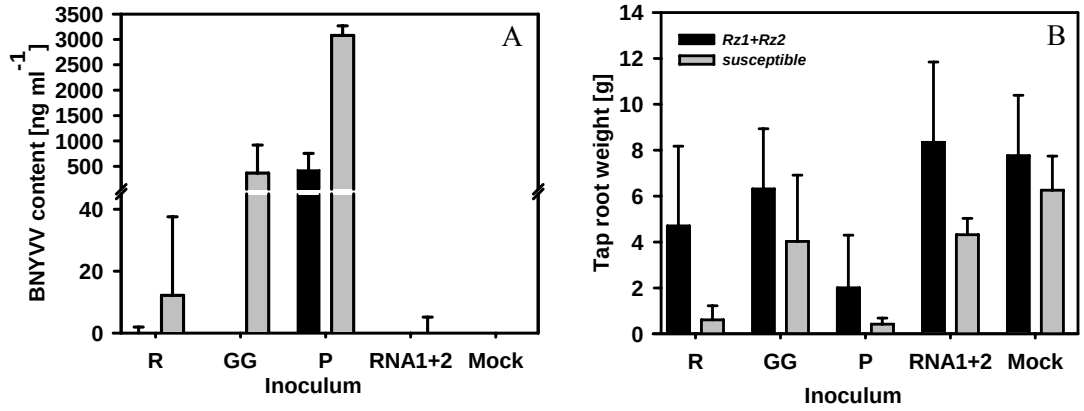

Fig. 4: BNYVV content in lateral sugar beet roots (A) and tap root weight (B) after vortex inoculation with 4 different BNYVV isolates as well as a non-infested Mock-control and cultivation for 12 weeks in greenhouse (R =

Rovigo – Italy; GG = Groß Gerau – Germany; P = Pithiviers – France; only RNA1+2 from an B-type isolate without the pathogenicity factor on RNA3).

A very strong negative correlation between tap root weight and BNYVV content in lateral roots of different sugar beet genotypes after vortex inoculation (KOENIG & STEIN, 1990) with wild-type BNYVV propagated on *C. quinoa* leaves was obtained (Fig. 4). For this artificicial inoculation 20 sugar beet seedlings were inoculated with 1:4 infected plant sap : 0.05M phosphate buffer (pH 7.2) by vortexing with 0.09 g carborund in a 2.2 cm diameter centrifuge tube. The seven-days-old seedlings were transferred into hydroponics for two weeks to recover from vortexing stress und thereinafter they were transplanted ino sterile soil. After 12 weeks under standardised green house conditions the sugar beet plants were harvested and the tap roots were weighed. Sugar beet hair-roots were separately harvested and analysed for BNYVV content via DAS-ELISA. Additionally, the systemic spread within sugar beet tap roots could be proven via Tissue Print Immuno Assay (TPIA) following KAUFMANN et al. (1992). Generally, this kind of resistance test is difficult to evaluate due to the fact that no adjusted inoculum concentration can be produced with *C. quinoa* plant sap. This led in these studies to non-reproducible results because of highly variable virus contents. But with regard to methodical approaches the plants differentiated well after 12 weeks cultivation time. Only the consistency in virus content after inoculating with various aggressive BNYVV isolates could not always be shown. However, if the harvest had have been at an earlier stage (4 to 8 weeks, respectively) the tap root would have been too small to display significant weight effects of virus infection. Moreover TPIA is quite difficult to conduct with very small tap roots. 12 week-old sugar beet plants have passed the physiological juvenile phase and have formed already a real tap root comparable to beet grown in practice. Thus beet weight and ELISA values are closer connected to realistic yield and ELISA performance under natural growing conditions. BÜRCKY & BÜTTNER (1991) already reported, that the virus content in partial resistant sugar beet rootlets is decreasing over the vegetation period in field trials, thus they suggested conducting virus quantification only from tap roots instead of lateral roots independent from cultivar resistance abilities. In these trials (manuscript **II**) similar observations of decreasing virus content could also be shown comparing harvests after 4, 8 and 12 weeks in artificially inoculated soil. With relation to a four weeks trial, where only lateral sugar beet roots are analysed in ELISA, it must be considered if the roots are developed enough for resistance testing at all. To date cultivar tests to show the performance in Rhizomania infested soils were usually conducted in naturally infested soil where overcoming of resistance has not yet been observed. The underlying resistance mechanisms as well as reasons for resistance breaking are still completely unknown, thus new set ups to test cultivars need to be developed in the face of testing more aggressive BNYVV isolates.

3.2. Overcoming of resistance depending on different BNYVV isolates

Resistance breaks in four different soils from D, IV MN and P concerning the *Rz1* cultivar were demonstrated in a 12 weeks trial (manuscript **I**). Results of relative virus content were also correlating to field trials and six weeks greenhouse tests (HEIJBROEK et al., 1999; LIU et al., 2005; RUSH et al., 2006; LIU & LEWELLEN, 2007; AYALA-GARCIA pers. comm.; LENNEFORS pers. comm.). However in the study described here, overcoming of resistance was not only documented by ELISA values obtained in lateral roots (which were invariably higher or at least similar to the ELISA contents measured in susceptible plants) but also by TPIA (manuscript **II**). Additional reduction of tap root weight indicated a higher BNYVV damage (manuscript **II**), but this weight decline could also be connected to different primary nutrition supply in various soils or by other soil-borne pathogens infecting the plants. The tap root weight was reduced significantly after 12 weeks cultivation time compared to weight values obtained in less aggressive soils from Italy (R) and Germany (GG) (manuscripts **I** + **II**). The reduction of tap root weight under severe BNYVV infestation in soil from D, IV, MN and P has not yet been demonstrated in a greenhouse before. In contrary to experiments published here, HEIJBROEK et al. (1999) displayed weighing results after 9 weeks cultivation period in naturally infested soils from GG, R and P. However, no significant differences were determined between the less aggressive Italian soil and the French BNYVV-P isolate concerning the Rizor and an *Rz1* sugar beet variety, although the P-isolate is known to be much more aggressive than the R-isolate due to its additional RNA5 encoded pathogenicity factor P26 (MIYANISHI et al. (1999) and TAMADA et al., (2003) concerning results shown in manuscripts **I** + **II**. It seems sensible to consult not only ELISA values but also tap root weight as an additional criteria regarding resistance compromising BNYVV-isolates in soil. Especially, as demonstrated in the experiments of this study (manuscripts **I** + **II**) ELISA values of lateral roots in susceptible beets contain less BNYVV than the *Rz1* plants. Inferential, the stronger the infection pressure is and thus lateral root damage due to the virus, the slower the secondary infection with BNYVV is, as not enough newly developing root cells are available for *P. betae* and BNYVV multiplication. To confirm results obtained with susceptible and *Rz1* genotypes in soils showing heavy virus contents and very low tap root weights a TPIA was conducted with heavily affected sugar beet to prove if the virus is also systemically distributed within the tap root. In soils from D, IV, MN and P the TPIA of susceptible and *Rz1* plants resulted in systemic virus spread within the whole tap root. Regarding the *Rz1+Rz2* partial resistant genotype significant weight reduction compared to the same cultivar grown in other soils could only be demonstrated in D. Interestingly, the presence of BNYVV in lateral roots could be detected in D-, IV-, MN- and P-soils but spread of BNYVV into the tap root was only observed again in D and P. Therefore, it can be concluded that BNYVV D-soil isolate is compromising *Rz1* but also *Rz1+Rz2* resistance in naturally infested soil under the greenhouse conditions applied here. BNYVV-P is distributed systemically in *Rz1+Rz2* tap roots

and thus able to multiply but significant weight reduction of the tap root was not observed in the double resistant genotype.

3.3. Influence of variable P25 composition on virus pathogenicity

On closer examination of the pathogenicity factor P25, the high variability of tested BNYVV isolates became obvious. Particularly the amino acid tetrad $aa_{67\text{-}70}$ as already published in SCHIRMER et al. (2005) showed high variability between different BNYVV-isolates (manuscript **I**). However the predicted relation between valine on position 67 (V_{67}) and resistance breaking was not confirmed. These results are supported by recently published data from LIU & LEWELLEN (2007), who did not find any correlation between a specific tetrad composition and overcoming of resistance. In the experiments shown here the most aggressive soil contained two different BNYVV isolates with the following tetrad compositions: ACHG and VCHG (manuscript **I**).

3.4. Phylogenetic analyses of *Polymyxa betae*

Besides virus content in lateral roots and composition of the viral pathogenicity factor analyses of the naturally occurring vector densities of *Polymyxa betae* should clarify if a higher vector concentration in soil is correlating to successful virus transmission and thus eventually to observed resistance breaks. In these experiments (manuscripts **I** + **II**) the MN soil was analysed for astonishing high content of secondary *P. betae* zoospores. All other soils vs. genotypes resulted in about 10 to 40 times less *P. betae* inoculum. As the BNYVV infestation of sugar beet in MN soil tends to decline compare to D, IV and P, the density of viruliferous zoospores must be marginal in MN. Further investigations concerning the percentage of viruliferous *P. betae* were conducted in manuscript **II**. Due to these highly variable results concerning *P. betae* contents in soil and the great geographical distance between different soil origins the internal transcribed spacer ribosomal DNA region (ITS1+5.8+ITS2) was amplified, sequenced and aligned for phylogenetic analysis to examine if *P. betae* diversifies in its genomic composition. WARD (1994) published that the ribosomal DNA has proven to be a suitable region to study these genomic diversities. This region contains genes like 5.8S and 18S that differ only marginally between species and other regions, such as the ITS region that is more variable (WARD et al., 1994; WARD & ADAMS, 1996; LEGRÈVE et al., 1998/2002). WARD & ADAMS (1998) and MORALES et al. (1999) conducted studies on ITS1, 5.8S gene and ITS2 sequences from *Polymyxa* isolates and described diverse subspecies of *P. graminis* that differed only minimal from *P. betae*. Therefore, LEGRÈVE et al. (2002) showed much larger diversity in *P. graminis* regarding ITS sequences of isolates from separate geographical origins. But as soon larger distinctions could be detected after completing phylogenetic analyses of *Polymyxa graminis* spp. groups were developed that always had the same

host but originated from totally different locations. Surprisingly, in manuscript **I** only very few nucleotide exchanges could be detected between sequenced *P. betae* ITS1+5.8+ITS2 from different countries and continents. Apparently deletions, insertions and other mutations, which could appear due to higher genomic diversification, were not detected and thus not correlated to its geographic spread. *P. betae* could be distributed via its host the sugar beet. Due to the same host world-wide a genomic adaptation to a changing environment was not necessary. Transferring this knowledge to *P. betae* means that the probability to mutate but still infecting the same host (the sugar beet) seems to be unlikely no matter if *P. betae* originates from Germany or the USA. It can be assumed that *P. betae* has been spread coeval with sugar beet production first within Europe, onwards from Europe to America and Asia not more than 100 – 150 years ago.

3.5. Other soil-borne fungal pathogens

Additional pathogens in various densities are always occurring in naturally infested soils (manuscript **I**). In some soils the inoculum density of soil-borne pathogens was so high that an additional effect due to primary or secondary infection by *Pythium* spp., *Fusarium* spp. and *Rhizoctonia solani* cannot be disregarded. In our studies (manuscript **I** + **II**) a plantlet treatment with Tachigaren (Hymexazol + Thiram) could not entirely prevent seedling infection by soil-borne fungi. Especially in D soil the inoculum potential of *Rhizoctonia solani* and *Pythium* spp. had an impact on plant development and disease severity. Experiments with artificial BNYVV inoculated seedlings transplanted into *Rhizoctonia solani*, *Fusarium* spp. or *Pythium* spp. infested soil could provide information if these diseases possess an impact on BNYVV symptom severity. However, experimental proof is lacking yet.

3.6. BNYVV and *Polymyxa betae* inoculum potential

3.6.1. Attempts for artificial infection with viruliferous P. betae

BNYVV and *Polymyxa betae* inoculum in soil can be either estimated via bait plant test (Most Probable Number test – MPN) with susceptible sugar beet cultivars in naturally infested soil accomplishing a serial soil dilution (TUITERT, 1990) or by polymerase chain reaction (PCR) (MUTASA et al., 1995, 1996, MUTASA-GOTTGENS et al. 2000; KINGSNORTH et al., 2003; WARD et al., 2004). However, in assays where *P. betae* was quantified directly from soil via (time-saving) real-time PCR (WARD, 2004) the number of *P. betae* units actually able to infect the sugar beet remained unknown. TUITERT (1990) used for the MPN soil from the Netherlands which is most likely to contain a BNYVV-A-type. The aim of the study was to determine the inoculum densities and the effect of adjusted inoculum under standardised greenhouse conditions from different BNYVV-types (manuscript **II**). BNYVV isolates which were known to be able to overcome *Rz1*

resistance in sugar beet in field or greenhouse trials (HEIJBROEK et al., 1999; LIU et al., 2005; RUSH et al., 2006; LIU & LEWELLEN, 2007; AYALA-GARCIAS pers. comm.; LENNEFORS pers. comm.) were investigated. To exclude the influence of other soil-borne pathogens, viruliferous *P. betae* zoospore suspensions in automatic immersion systems were developed (ADAMS et al., 1988; LEGRÉVE et al., 1998) from *P. betae* isolates originating from R, GG, D, IV, P as well as *P. betae* that remained virus-free (vf). To obtain single spore *P. betae* suspension, cystosori developed from infected sugar beet lateral roots were selected with a micromanipulator and incubated in the dark for 24 h at room temperature in 0.05 M phosphate buffer (pH 7.2). The suspension was replenished with nutrient solution (pH 7.2) in an automatic immersion system (LEGRÈVE et al., 1998), wherein susceptible sugar beet plantlets were transplanted in silica sand filled poly-vinyl-chloride culture tubes. The tubes were closed at the bottom side with a mesh that avoid the silica sand to fell into the hydroponics but still offering the *P. betae* zoospores the opportunity to infect the sugar beet. By irrigating the filled tubes every 6 h for 6 h time the *P. betae* zoospores were able to enclose and swim to the sugar beet rootlets to propagate. The hydroponics-solutions were replaced each week, until the plantlets were incubated three weeks. Thereinafter the lateral roots were harvested and under dark and warm (25-30°C) conditions the *P. betae* zoospores were evolving from the lateral roots. The amount of living zoospores were counted by light microscopy in a Fuchs-Rosenthal haemocytometer and the suspensions were diluted down to 100 and 1000 zoospores per ml, respectively. Afterwards susceptible and *Rz1+Rz2* partial resistant sugar beet cultivars were infected with 100 and 1000 zoospores ml^{-1}, respectively. The inoculation method followed SCHOLTEN et al. (1994) inoculating with different *P. betae* carrying various BNYVV isolates. For analyses of single beet grown in different zoospore concentrations a qualitative ELISA after four weeks of cultivation in zoospore suspension was conducted. After analysing the data by using SAS 10.0 (SAS Systems, Cary, NC) with the PROC GLM procedure the data resulted to be normal distributed but the sugar beet cultivars often did not differ significantly concerning the zoospore density and their resistance potential (Fig. 5).

Fig. 5:

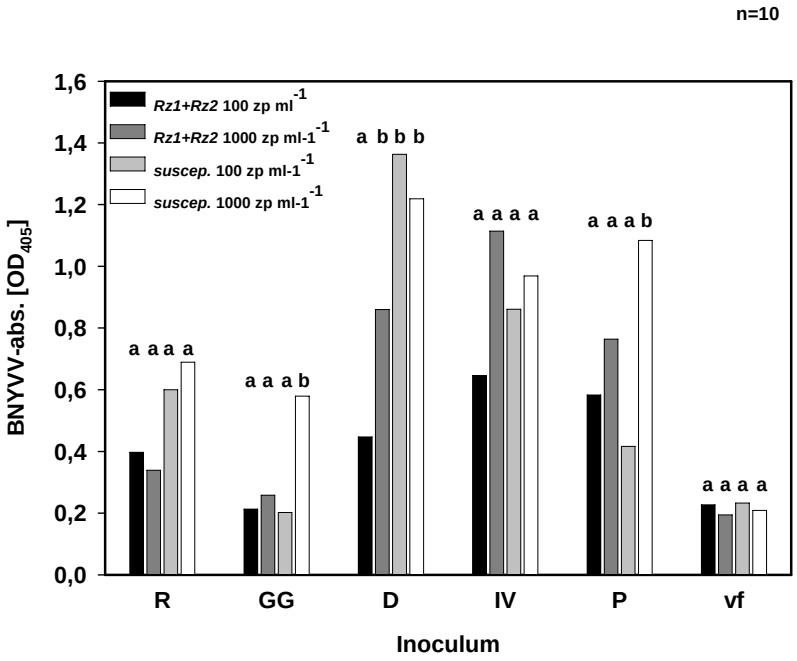

Fig 5: Means of BNYVV ELISA absorption at 405 nm after 4 weeks seedling cultivation in hydroponics containing either 100 *P. betae* zoospores per ml (zp ml^{-1}) or 1000 *P. betae* zoospores per ml originating from Rhizomania infested soils from R (Rovigo – Italy), GG (Groß Gerau – Germany), D (Daimiel – Spain), IV (Imperial Valley – USA), P (Pithiviers – France) as well as an virus-free (vf) *P. betae* control originating from Reutershof (Germany). Means within the same inoculum with a letter in common are not significantly different at the 5% level.

In P and IV the partial resistant cultivar obtained higher virus titres than the susceptible genotype. After several efforts to repeat the experiment only very low or no infestations with BNYVV were observed, whereas successful root infection of *P. betae* could be proven by light microscopy in all plantlets. Thus, it can be concluded that *P. betae* is probably loosing the virus or it forfeits the ability to transmit the virus into the sugar beet when propagated in hydroponics. Consequently, after adjusting the amount of secondary zoospores to conduct resistance tests with a defined vector inoculum dose as presented in SCHOLTEN et al. (1994), the actual BNYVV transmission and sugar beet infection is absolutely unknown. In addition, contamination with other pathogens could not be excluded although using hydroponics generated from single-spore suspensions. Thus, to optimise the zoospore culture the hydroponics need to be completely contamination free. But still the amount of viruliferous *P. betae* would stay unknown, what leads to unidentified BNYVV-inoculum dose. Further efforts to develop virus-free *P. betae* zoospores to load them with artificially BNYVV-infested sugar beets and thereinafter inoculate again with defined *P. betae* zoospores however cannot exclude the problem of inoculating with variable BNYVV-inoculum densities, since the percentage of viruliferous zoospores is highly variable.

3.6.2. BNYVV and P. betae inoculum density in naturally infested soils

Due to the unreliable results from the artificial inoculation with viruliferous *P. betae* a primary determination of the BNYVV and *P. betae* inoculum potential in all naturally infested soils used (R, GG, D, MN, IV, P) was necessary. Secondary it was possible to estimate the percentage of viruliferous *P. betae* and latter to accomplish a resistance test with *Rz1+Rz2, Rz1* and susceptible sugar beet cultivars with adjusted inoculum in sterile soil (manuscript **II**).

The MPN (manuscript **II**) revealed significant differences between soil origins (5200 in D and only 11 in GG) with susceptible cultivars that redound to conduct the same serial soil dilution also with a *Rz1+Rz2* partial BNYVV resistant genotype. By measuring the BNYVV content in *Rz1+Rz2* sugar beet the effect of primary inoculum potential in soil could be tested and compared to common MPNs with susceptible genotypes. Displaying that even in high dilutions, up to a ratio of 5^{-4} in D, partial resistant sugar beets were infected with BNYVV. Primary inoculum potential in soil cannot just underlie resistance breaks, although, a high primary inoculum potential as demonstrated in D, seems to enhance further virus propagation. Taking the same into account for MN, which displays a comparable low BNYVV MPN (98), it also infects resistant plants when the soil is diluted down to 1 / 625 (infested soil / sterile sand). Thus, BNYVV multiplication is operating much more sufficient than in D. In reference soils, as R and GG, BNYVV is restrained latest in the second dilution step concerning the *Rz1+Rz2* genotype. This indicates that the infestation of BNYVV is inhibited in less aggressive Rhizomania infested soils like R and GG when the inoculum dose is low and resistance in sugar beet maintains. To test the influence of specific primary inoculum under standardised soil and climate conditions, finally a resistance test was conducted in which the inoculum was added to sterile sand by dried sugar beet rootlets (manuscript **II**). Three harvests were carried out: after 4 weeks, after 8 weeks and finally after 12 weeks. ELISA values and sugar beet weight differed significantly. It was obvious that tap root weight did not differ after 4 weeks within the genotypes and BNYVV-origin. After 8 weeks the tap root weight differed moderately between BNYVV-origin and very little in between genotypes within the same BNYVV-origin (data not shown). Comparing both, the resistance test in natural infested and the resistance test in artificially inoculated soil, the results are closely correlated regarding ELISA values, tap root weight and virus distribution in the tap root via TPIA. Thus, quantitative ELISA and tap root weight after 12 weeks cultivation with an adjusted BNYVV dose in sterile soil are still the best criteria to test sugar beet cultivars for their resistance potential. Reduced experimental time also result in differential data (PAUL et al. 1992; TUITERT et al., 1994; BÜTTNER et al., 1995; SCHOLTEN et al. 1996; HEIJBROEK et al., 1999; LIU et al., 2004;LUTERBACHER et al., 2005; LENNEFORS, 2007;). However, the ability of resistant sugar beet to recover from BNYVV infestation is not considered.

3.7. Genetic variability of BNYVV and its relation to virus spread

Interestingly, the German soil with the lowest potential to produce severe BNYVV symptoms harbours an BNYVV B-type which displays the least variability regarding its pathogenicity factor P25 (SCHIRMER et al., 2005). Whereas soils that recently displayed resistance breaks when partial resistant sugar beet were cultivated are always BNYVV-A-types, which are known to display higher genomic variability regarding the RNA3. Thus, overcoming resistance could be correlated to the higher variability in the virus genome. Apparently the A-type virus does frequently mutate in some locations as observed in Spain and the USA. This could also imply that the occurrence of resistant breaking isolates increases. In contrary the French BNYVV-P-type that contains an additional RNA5 and is known to be able to overcome *Rz1* resistance for almost two decades shows much lower P25 variability. However, the BNYVV-P-type did not spread further from a small district around Pithiviers also known to be more aggressive than the neighbouring BNYVV-A-type isolates all around this Pithiviers area. It can be speculated that soils infested with BNYVV-A-type vectoring *P. betae* are already saturated with the virus and multiply even in partial resistant cultivars, thus the further spread and establishment of BNYVV-P-type is inhibited. Similar thoughts are also published by WARD et al. (2007).

3.8. Infiltration of BNYVV-P25 + mRFP into sugar beet leaves

To shorten time-consuming resistance tests for practical application several experiments were conducted to co-infiltrate a BNYVV RNA3 encoding P25 infectious cDNA clone and a red fluorescing marker gene (mRFP) on sugar beet leaves. To achieve this, P25 and mRFP genes were cloned into the binary plant vector pBIN61S. Additional, the binary vector carried Kanamycin (Ka), Rifampicin (Rif) and Tetracyclin (Tc) resistance to survive on selective LB-Ka-Rif-Tc media. *A. tumifaciens* were incubated for 48 h at 28°C. After further 24 h incubation the optical density was measured and diluted to an optical density (600nm) of 0.5 to 0.6. The infiltration was accomplished with a 2 ml syringe. To characterise BNYVV pathogenicity, P25 (35S-P25) was expressed together with mRFP (35S-mRFP) by transient *Agrobacterium tumefaciens* in leaves of susceptible, *Rz1* and *Rz1+Rz2* partial resistant sugar beet cultivars. Expressions could already been seen at 5 days post inoculation (dpi) using epifluorescence microscopy. It was expected to be able to show cell-death as a consequent of a resistance reaction and subsequently a decrease of fluorescing cells at 8 dpi in leaves of resistant sugar beet. CHIBA et al. 2003 showed, subject to the tetrad composition on P25, a hypersensitive reaction (HR) after sugar beet leaf inoculation. Unfortunately, following the reduction of marker expression due to cell death at approx. 8 dpi, cell-

death revealed to be independent from BNYVV resistance in sugar beet cultivars (Fig. 6). Thus, this approach to simplify and cheapen labour intensive resistance tests in soil failed.

Fig. 6A

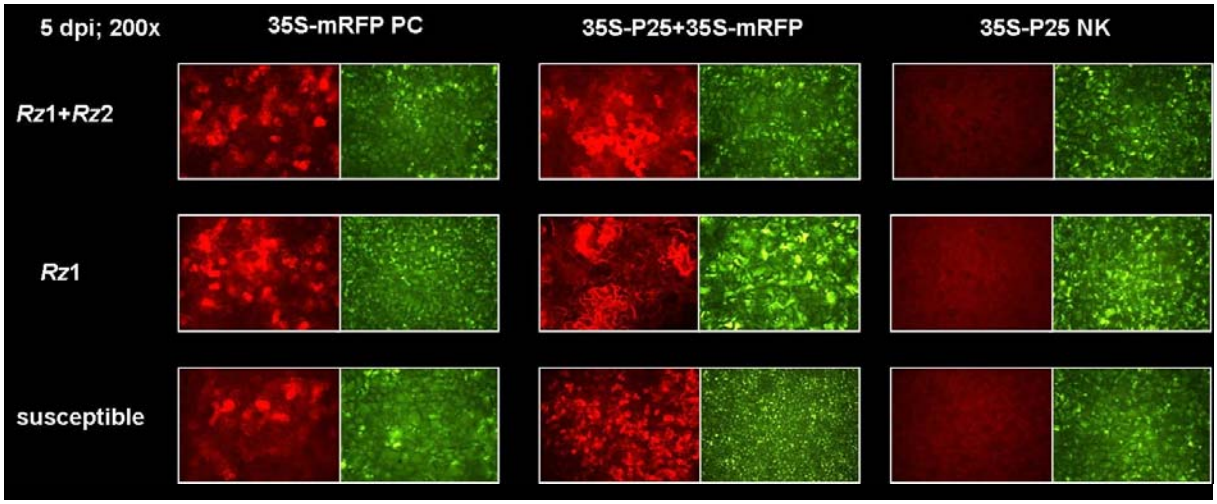

Fig. 6B

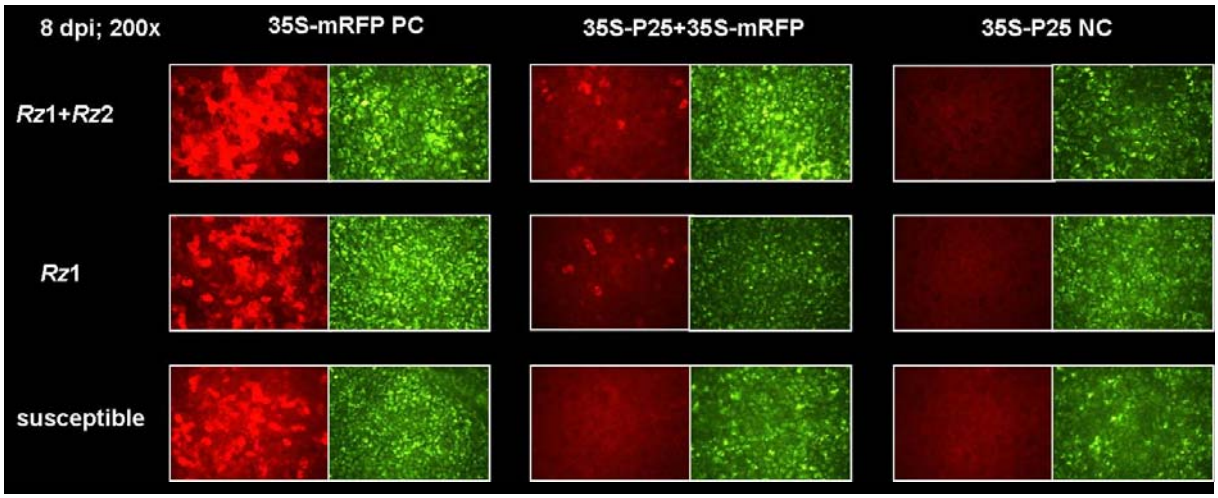

Fig. 6: Pictures made by epifluorescence microscopy with an mRFP-filter (red) of BNYVV susceptible, Rz1 and Rz1+Rz2 partial resistant sugar beet leaves after 5 dpi agroinfiltration (A) and 8 dpi agroinfiltration (B) with, 35S-mRFP as positive control (PC), BNYVV-P25 (35S-P25+35S-mRFP) both including the vital-marker (fluorescent marker gene mRFP) as well as a negative control (NC)- 35S-P25 without the vital marker to display background fluorescence. To prove the vitality of leaf pictures via light microscopy (green) with equal resolution has been taken..

Against the background of:

i) the uncertainty of virus-vector multiplication in soil by growing "Holly"-material in the field,

ii) the insecurity if and when resistance-breaking isolates are spreading in large scale to further sugar beet growing areas,

iii) the general concern, how long natural derived resistant genes like *Rz1, Rz2* and *Rz3* will be able to control BNYVV infestation in sugar beet and the

iv) knowledge of additional diseases occurring in soil that could play a synergistic role with BNYVV after sugar beet infestation

further investigations on BNYVV, its vector and the soil where the isolates are present are indispensable.

4. CONCLUSIONS AND FUTURE PROSPECTS

The main conclusions achieved in these studies are that

i) *Rz1* resistance in sugar beet is compromised in soils from Spain, the USA and Pithiviers

ii) the vector *P. betae* did not differentiate in a specific genomic compositions that would indicate evolutionary development after geographic distribution of Rhizomania to various sugar beet productions areas. Vector variability could have been correlated to overcoming resistance.

iii) the vector concentration in the soil is very variable with regards to different soil origins

iv) other soil-borne pathogens (viruses and fungi) are playing an important role in respect of disease severity. However, experimental evidence is yet lacking.

In addition the results obtained give strong evidence that

v) there is a high variability in BNYVV content in soils from different origin,

vi) most soil samples (Spanish, American and French soil) analysed in this study which display the ability to produce BNYVV infections under controlled conditions of a certain titre, possess an increased inoculum concentration but

vii) the potential to overcome resistance is not correlated to the inoculum concentration

viii) neither the *P. betae* concentration itself nor the concentration of viruliferous *P. betae* is invariable correlated to resistance-breaking.

Apparently, all above mentioned factors as well as genetically related aggressiveness leads to overcoming of *Rz1* and *Rz1+Rz2* resistant genotypes in greenhouse and in field trials. To be able to prevent Rhizomania infestation and resistance breaks in field, the selection of new natural occurring resistance sources is indispensable. Additionally sugar beet cultivars with high multiple resistance against BNYVV and different soil-borne pathogens should be developed. Transgenic sugar beets obtaining these multiple resistances are good approaches, but consumer and political acceptance is not attained, yet. Further is the better understanding of virus-host interaction very important to be able to analyse why resistance breaks occur with several BNYVV isolates in different genotypes. Detailed information about the virus-vector interaction needs also further investigation. For sugar beet growers it would be important to get knowledge on the level of resistance in marketable sugar beet cultivars to be able to inhibit supplementary virus-vector accumulation in soils.

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APPENDIX

Summary of Manuscripts I and II

Manuscript **I** presents resistance tests in naturally infested soils after 12 weeks greenhouse cultivation. Thereinafter beet weight, virus content in lateral roots (ELISA) and virus distribution (TPIA) were analysed. Additional different BNYVV isolates were molecular characterised and beets were investigated for secondary fungal soil-borne pathogens. Since the resistance test resulted in huge differences concerning soil vs. sugar beet genotypes, the BNYVV inoculum density of each soil was estimated by MPN (manuscript **II**). The same serial soil dilution as accomplished for the BNYVV MPN calculation with susceptible cultivars was done with *Rz1+Rz2* partial resistant genotypes. Since the vector density plays an important role for successful virus transmission, *P. betae* inoculum density was estimated, too. Subsequently, the virus-vector performance after artificially infected sterile soil with a defined number of viruliferous *P. betae* was analysed. Different stages of *P. betae* and BNYVV multiplication could be described due three harvest dates (4, 8 and 12 weeks).

MANUSCRIPT I

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

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Abstract

Rhizomania, caused by beet necrotic yellow vein virus (BNYVV) is vectored by *Polymyxa betae* Keskin. It can only be controlled by growing partial resistant sugar beets which quantitatively reduce the virus replication and spread. However, none of the known major resistant genes (*Rz1, Rz2, Rz3*), alone or in combination, is able to prevent BNYVV infection entirely. Hence, a permanent increase of BNYVV inoculum potential in soil seems to be inevitable. We are reporting here for the first time about the identification of a European soil, containing A-type BNYVV with RNA1-4 which displays *Rz1* resistance breaking abilities comparable to soils from the USA and RNA5 expressing BNYVV French P-type. First, under standardised conditions, a resistance test with several soils vs. sugar beet cultivars was conducted. Sugar beets were analysed after 12 weeks greenhouse cultivation for tap root weight, virus and relative Polymyxa content. Soils from Spain, France and the USA displayed the ability to overcome *Rz1* resistance. The *Rz1+Rz2* cultivar grown in soil from Spain displayed strong Rhizomania disease symptoms. Additionally, the main pathogenicity factor P25, which is responsible for the formation of BNYVV-symptoms, revealed high sequence variability with regard to the amino acid tetrad at position 67-70. No correlation of P25 tetrad composition and resistance breaking abilities were found. The results demonstrate the

geographically independent selection of BNYVV resistance breaking isolates after uniform cultivation of *Rz1*-containing sugar beet cultivars.

Key words: sugar beet, *Beta vulgaris* L. ssp. *vulgaris*, Beet necrotic yellow vein virus, BNYVV, *Polymyxa betae,* resistant cultivars, *Rz1*, *Rz2*

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 (Canova, 1959) to be the causal agent for severe yield reduction in sugar beet. Analogue to other soil-borne pathogens as beet soil-borne virus (BSBV), beet soil borne mosaic virus (BSBMV) and beet virus Q (BVQ), BNYVV is transmitted by the biotrophic plasmodiophoromycete *Polymyxa betae* Keskin (Keskin, 1964; Tamada, 1975; Ivanović et al., 1983; Abe & Tamada, 1986; Wisler et al., 1994; Stas et al., 2001). While the vector itself hardly affects sugar beet growth (Rush, 2003), severe symptoms on foliage (fluorescent yellow) and drastic reduction of tap root development are induced by the virus (Rush & Heidel, 1995; Tamada, 1999; Scholten & Lange, 2000; Acosta-Leal & Rush, 2007). Over the last decades, BNYVV was spread to all important sugar beet growing areas in Asia, USA and Europe (Asher, 1993; Tamada, 1999; Lennefors et al., 2000; Nielsen et al., 2001). A permanent increase of Rhizomania infested fields in Europe is expected; Richard-Molard & Cariolle (2001) calculated an augmentation 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. It typically consists of four to five RNA segments in the field. The genome organisation and gene functions of each RNA segment have been recently reviewed by Schirmer et al. (2005), Link et al. (2005) and Rush et al. (2006). Molecular analysis of the BNYVV genome disclosed three major pathotypes (Kruse et al., 1994; Koenig & Lennefors, 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 appears in Germany, in the upper Rhine valley, in France and in Switzerland. 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 pathotype that contains an additional RNA5, A and B possess four RNA segments (Koenig et al., 1986; Richards & Tamada, 1992). In Asia BNYVV isolates (BNYVV-J) with an additional RNA5 were identified which display sequence variability to French P-type as well as to the classical A- and B-types (Koenig & Lennefors, 2000; Schirmer et al. 2005). Compared to BNYVV-A and -B, a BNYVV-P isolate, containing RNA5, is characterised by its

more rapid spread in plants (Heijbroek et al., 1999). Tamada et al. (1996) identified increased virulence of Asian RNA5 containing BNYVV isolates in sugar beet roots. RNA3 encoded P25 is highly variable, acts as the main pathogenicity factor and is responsible for BNYVV-symptoms on sugar beet roots and development of necrotic lesions on *Chenopodium quinoa* leaves (Koenig et al., 1991; Jupin et al. 1992; Schirmer et al., 2005). Phenotypic local lesions on mechanical inoculated sugar beet leaves are depending on P25 composition and vary highly within different BNYVV isolates and resistance sources (Tamada et al., 1989; Chiba et al., 2003). Vetter et al. (2004) described P25 as a nucleoplasmic shuttling protein and demonstrated that the *C. quinoa* local lesion phenotype is influenced by the subcellular localisation of the protein. P25 displays high variability in a specific amino acid (aa) tetrad at position 67-70 ($aa_{67\text{-}70}$) which correlates with the BNYVV geographic origin (Schirmer et al., 2005). Link et al. (2005) detected the RNA5 encoded 26kDa protein P26 that serves as additional pathogenicity protein. It can be assumed that P25 acts in a synergistic manner with P26 (Tamada et al., 1996; Link et al., 2005). Yet, severe sugar beet yield losses could only be prevented by growing partial resistant genotypes. However, for keeping sugar beet growth profitable, BNYVV resistant and high-yielding cultivars are necessary. The first partially resistant sugar beets were developed in the mid 1980s, the variety "*Rizor*" showed significant partial resistance and achieved good yield improvements under Rhizomania infection compared to yields in non-infested soil (De Biaggi, 1987). Little later Lewellen et al. (1987) reported partial 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 & Lange, 2000 and Biancardi et al., 2002). Additional, wild beet (WB) accessions served as further sources for the identification of other major resistance genes, such as *Rz2* and *Rz3* resistance genes (Whitney, 1989; Scholten et al. 1994 / 1999; Gidner et al., 2005). However, all known major resistant genes, alone or in combination, provide only partial resistance and are unable to prevent BNYVV infection entirely. *Rz1*, *Rz2* and *Rz3* only possess the ability to reduce the virus replication in hair roots and inhibit virus spread to the tap root (Luterbacher et al., 2005). Thus, a permanent increase of BNYVV inoculum potential in soil, although cultivating partial resistant sugar beets, seems to be inevitable.

In soils from the USA BNYVV A-type isolates occurred, that were able to infect cultivars carrying *Rz1* as well as *Rz1+Rz2* resistance genes (Liu et al., 2005, Liu & Lewellen, 2007). Within these isolates a P25 aa motif with valine instead of an alanine on position 67 was determined, but evidence of P25 $aa_{67\text{-}70}$ composition effect on pathogenicity in sugar beet is lacking. In addition, the influence of the vector *P. betae* (density in soil, ability to transmit BNYVV, mobility in soil etc.) is unknown.

Recently in Spanish variety trials severe BNYVV symptoms were observed in resistant cultivars (Ayala, pers. comm.). In order to prove the resistance breaking abilities, this soil was included in a resistance 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 of beets grown in these soils was quantified by ELISA. To study a possible effect of *P. betae* its concentration in plants and its genomic composition regarding the ribosomal DNA internal transcribed spacer (ITS) region were determined. The ITS region is often variable in fungal isolates of a single genus and in some cases in isolates of a single species (White et al., 1990) and allows to determine phylogenetic diversity to possibly resolve the *P. betae* taxonomy from different origins. To identify additional soil-borne pathogens that may affect sugar beet growth and BNYVV content in lateral hair roots through synergism with BNYVV the ITS region of fungal isolates recovered from beets grown in the different soils analysed was determined.

Materials and methods

Plant material

Different sugar beet cultivars and lines were used for BNYVV greenhouse resistance tests towards various BNYVV-types in naturally infested soils: a BNYVV-double resistant variety (*Rz1rz1+Rz2rz2,* referred to as *Rz1+Rz2),* a single-resistant variety (*Rz1rz1,* referred to as *Rz1*) (Liu et al., 2005) and a susceptible sugar beet line (*rz1rz1*) as a control. The *Rz1rz1* resistance is also known as the Holly source, described in 1987 by Lewellen et al. selected from *B. vulgaris* subsp. *vulgaris*, whereas *Rz2rz2* originates from the *B. vulgaris* subsp. *maritima* accession WB42 (Whitney, 1989).

Soil samples and greenhouse conditions

Soil samples used for resistance tests in naturally infected soils, collected from different locations in Europe and the USA, were evaluated for disease induction in greenhouse tests. Soil samples from Italy (Rovigo, referred to as R), Germany (Groß-Gerau; GG), Spain (Daimiel; D), USA (Imperial Valley - IV and Minnesota - MN), France (Pithiviers; P) and an autoclaved sand control were diluted in equal parts with autoclaved sand and mixed thoroughly (for 10 min). 700 ml plastic pots were filled with 100 ml clay granulate for drainage conditions and topped up with 650 g soil sample. To avoid contamination between different soils, pots were arranged soil-wise 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 placed at least 30 cm apart to

avoid contamination by splashing during irrigation. Seven days old seedlings, previously sown in sterile sand, were transplanted into fully water saturated soil. Seven days after planting the beets were sprayed with fungicides (0.2 g/l Tachigaren 70, registered trade mark, Bayer, Germany) to stem damping-off due to *Aphanomyces* spp., *Pythium* spp. and *Rhizoctonia* spp. The climate chamber was maintained at +23°C during daytime and +20°C at night, and 16h of supplementary light.

BNYVV ELISA detection and quantification

After 12 weeks of greenhouse cultivation sugar beets were harvested individually. Beets were carefully taken out of 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 hypocotyledone and discarded. Fresh beet bodies were individually weighed and shape and discolouration was recorded and scored from 0-9. The complete absence of BNYVV-symptoms was recorded as 0 whereas 9 displays a heavily BNYVV infected plant displaying typical severe Rhizomania symptoms (a small "T-like" tap root with brownish vasculature and dark brown lateral roots, as well as yellowing of leaf veins). For BNYVV ELISA plant sap from hair roots of each beet was obtained via a Pollähne leaf juice press (MEKU GmbH, Germany) and PVP-Tween-buffer and diluted 1:15 in extraction buffer. 96 well ELISA plates were used from Nunc A/S, Roskilde Denmark. Besides blanks and buffer-controls, the following control samples were included: 2 samples of each, healthy and infected plant sap, respectively and an additional dilution series from a *Chenopodium quinoa* BNYVV- purification. The total virus protein concentration of the purified BNYVV preparation was determined using the Bradford assay (Perkin Elmer Instruments; Lambda 25 UV VIS Spectrometer, USA). Protein contents in samples were adjusted to 4000 ng/ml and aliquots kept at -20°C. The dilution series for generation of a standard curve consisted of 4000, 2000, 1000, 500, …, 1,95ng virus protein / ml buffer. The BNYVV detection limit (0 ng ml^{-1}) resulted from a mean of tested healthy controls plus three times the standard error. Antibodies used for ELISA detection were obtained from Loewe (Sauerlach, Germany) and DSMZ (Braunschweig, Germany) and the assay was carried out following the manufacturers' instructions. The absorption at 405nm (abs_{405nm}) was measured using a Titertek Multiskan Plus (Magarete Malar, Nauheim, Germany) after an incubation period of one hour at 37°C.

Polymyxa betae quantitative ELISA

Beet samples harvested from each soil origin were visually examined for presence of *P. betae* zoospores and cystosori through microscopy before *P. betae* quantitative ELISA detection was conducted. Specific detection of *P. betae* zoospores was achieved with mono- and polyclonal antibodies against recombinant expressed fungal glutathione-S-transferase (GST) as described by Kingsnorth et al. (2003) with slight modifications. All incubation steps of the *P. betae* TAS-ELISA were performed for 1 h at 37°C. As alkaline phosphatase tagged antibody, the whole molecule anti-rabbit antibody (IgG Sigma A3937) was used. To quantify the expressed glutathione-S-transferase (GST) from *P. betae*, a standard curve with purified GST (Broom´s Barn, England) dilutions series with the ratio 2 from 2048 to 1, respectively, was prepared and analyzed in parallel. Total protein concentration of the GST standard was determined using the Bradford assay as already mentioned above. The highest concentration (abs_{405nm} 1.2, calibration 2048) refers to a relative *P. betae* protein ratio of $GSTconc._{log}3000$ the calibration 1 (abs_{405nm} 0.007) displays a $GSTconc._{log}0$, respectively.

BNYVV RT-PCR detection and determination of BNYVV type / P25 sequencing

Total RNA was extracted from sugar beet lateral roots derived from the resistance test using RNeasy (Qiagen, Hilden, Germany) and used for RT-PCR amplification of RNA-3 encoded P25 RNA. Primers P25-up (5´-TCGGAATATCCATTTAAAAG-3´) and P25-low (5´-GTCCCAACCAGATC AACAA-3`) designed on BNYVV RNA-3 B-type sequence (Acc. no. M36894) amplified a 906 bp fragment (nt. 302 – 1207). The following PCR program 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 visualized on agarose gels and sequenced without further cloning to detect mixed infections of BNYVV with P25 sequence variants.

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 (1g) were N_2-liquid frozen and disrupted to a fine powder in lysis buffer (400 mM Tris-HCl [pH8.5], 60 mM EDTA [pH 8.5] 150 mM NaCl, 1% sodium dodecyl sulphate) using mortar and pestle. All centrifugation steps were conducted at 11000 x *g* for 5 min at 4°C. DNA was used for Polymyxa specific nuclear ribosomal DNA amplification of a fragment of 454 bp between the ITS1, 5.8 gene and the ITS2 region from

P. betae using primers Psp1 and Psp2rev as described by Legrève et al. (2003) [Psp1 (5´-TAGACGCAGGTCATCAACCT-3´) and Psp2rev (5´- AGGGCTCTCGAAAGCGCAA-3´)]. To determine Polymyxa specific sequences of this region, the PCR-products were cloned and sequenced using standard primers.

Identification of other soil-borne pathogens

To analyse the presence of other soil-borne pathogens, that might have influenced the sugar beet growth due to secondary infection, tissue from the inside of sugar beet tap roots displaying root rot symptoms were surface sterilized with EtOH and incubated on Potato dextrose agar (PDA, Roth, Karlsruhe, Germany) +150 mg/l Streptomycin Applichem, Darmstadt, Germany) for isolation of fungal pathogens. From each genotype vs. soil variant 5 beets were chosen to analyse any additional soil-borne pathogens. After 9 days the outgrown mycelia was grouped using morphological criteria. In addition, total DNA extraction (DNeasy, Quiagen, Hilden, Germany) was performed and extracts were subjected to PCR amplification of ribosomal DNA with ITS4 and NS7 primer containing the 3´-end of the 18S, the 5.8S gene and two internal transcribed spacer ITS1 and ITS2 (White et al., 1990). PCR fragments were directly sequenced with NS7 primer. For sequencing PCR-products were purified with SureClean (Bioline, Mannheim, Germany) following the manufacturers protocol. Sequencing was done 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.

Sequencing

DNA sequences obtained by PCR from samples originating from each soil sample were used to test for polymorphisms in a region that corresponds 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). *P. betae* sequence [GenBank Acc. no. Y12827 (Ward & Adams, 1998)] was used for sequence comparison of *P. betae* from different geographical origins. Additional 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)].

Phylogenetic trees

In this study, DNA sequences obtained by PCR from samples originating from each soil sample were used to test for polymorphisms in a region that corresponds 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). *P. betae* sequence [EMBL accession no. Y12827 (Ward & Adams, 1998)] was used for sequence comparison of *Polymyxa betae* from different geographical origins. Additional sequences of several *Polymyxa graminis* subspecies and other plasmodiophorids were taken for sequence comparison [*P. graminis* f. sp. *temperata* (EMBL accession no. Y12824), *P. graminis* f. sp. *tropicalis* (EMBL accession no. Y12825), *P. graminis* f. sp. *colombiana* (EMBL accession no. AJ010424), *P. graminis* f. sp. *tepida* (EMBL accession no.Y12826), *Ligniera* sp. (EMBL accession no. AJ010425), *Plasmodiophora brassicae* (EMBL accession no. Y12831)].

Data analyses

The data were analysed using SAS 10.0 (SAS Systems, Cary, NC). The PROC GLM and an unvaried procedure (PROC UNIVARIATE) to test for normality were conducted. A boxcar macro transformation (Anonymous, 2007) followed until all data displayed a normal distribution. The ANOVA was conducted with transformed data PROC MIXED procedure. The data are presented in the reverted transformed form.

Results

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 affect plant growth. After 12 weeks cultivation, the fresh harvested tap roots (Fig. 1) were weighed (Fig. 2) and scored for root discolouration due to infection with viruliferous *P. betae* (Tab. 1). An extension of the test period from four (data not shown) to twelve weeks led to more significant differences between the different genotypes. In all soils the susceptible genotype displayed significantly lower beet weights compared to the non-infected (sterile) sand control (Fig. 2). Especially sugar beets grown in soil from D only accomplished an average weight (aw) of only 0.27 g, IV 1.04 g, MN 0.75 g and P 0.9 g, respectively and displayed severe weight reduction.

Fig. 1

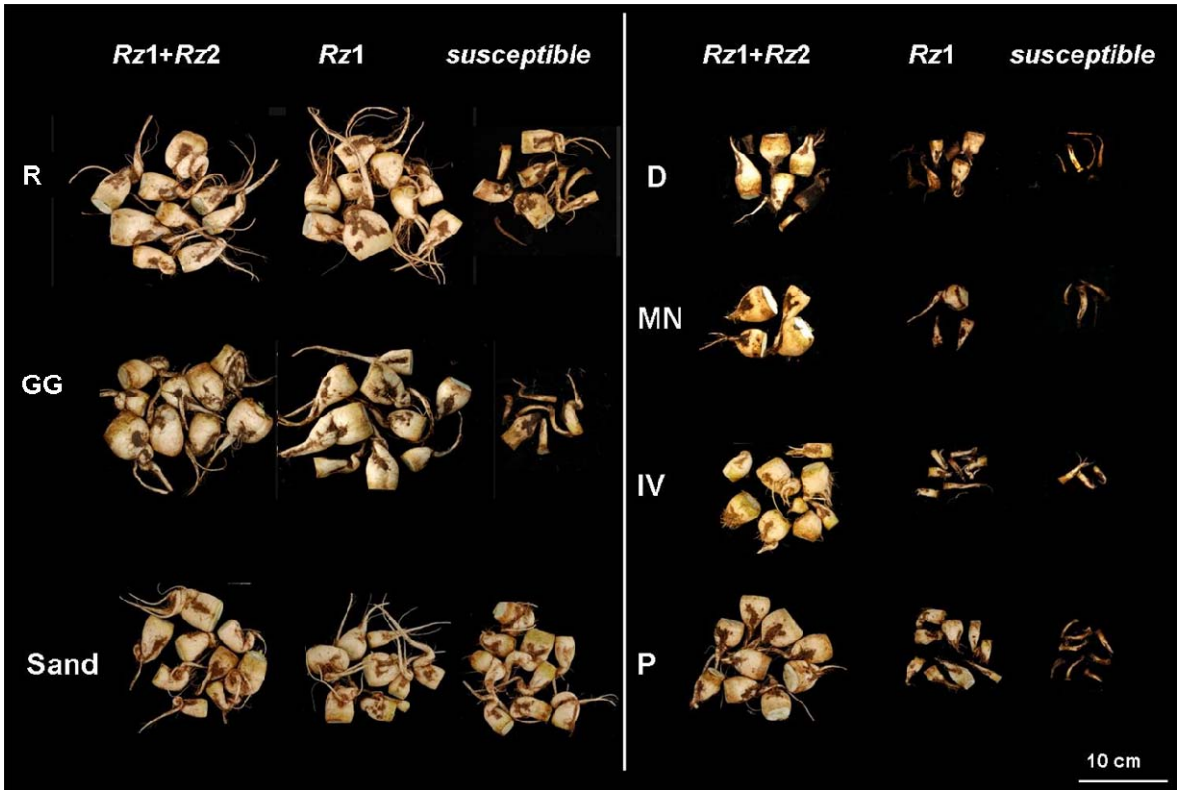

Fig. 1: Phenotypic appearance of harvested sugar beet bodies (with different resistance towards BNYVV: *Rz1+Rz2; Rz1* and *susceptible* genotypes) after 12 weeks greenhouse cultivation in Rhizomania infested soils from R (Rovigo/Italy), GG (Groß Gerau/Germany), D (Diamiel/Spain), MN (Minnesota/USA), IV (Imperial Valley/USA), P (Pithiviers/France) and an autoclaved sand control.

Fig. 2

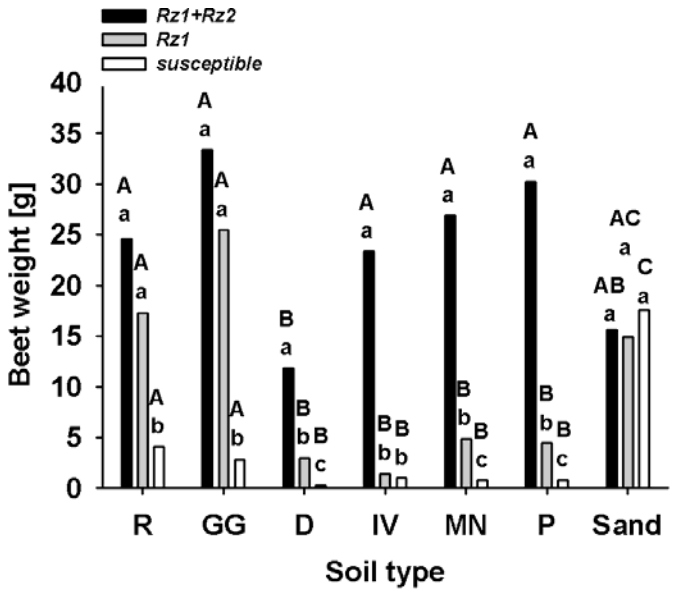

Fig. 2: Average beet body weight from different sugar beet cultivars (black: *Rz1+Rz2*; grey: *Rz1* and white: *susceptible*) after 12 weeks greenhouse cultivation in infested Rhizomania soils from R (Rovigo/Italy), GG (Groß Gerau/Germany), D (Diamiel/Spain), MN (Minnesota/USA), IV (Imperial Valley/USA), P (Pithiviers/France)) and an autoclaved sand control. Means within the same soil type (minuscule) and means within genotypes (capital) with a letter in common are not significantly different at the 5% level.

In contrast, 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 strong as the soils mentioned above. *Rz1* beets grown in R- and GG-soils were hardly affected by BNYVV, on the contrary, aw was increased 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, immense root weight reduction was observed for this sugar beet cultivar in soils from D (2.93 g), IV (1.40 g), MN (4.81 g) and P (4.47 g). Furthermore, the same kind of beet discoloration indicative for additional infection with fungal root rotting pathogens was observed as in the susceptible genotype (Fig. 1). 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 (6.93 g) and P (30.21 g), obtained higher aw than plants grown in sterile sand. Only *Rz1+Rz2* sugar beets in D-soil gained slightly less root weight (11.82 g) than plants grown in sterile sand and several beets showed discoloration and rotten tissue. BNYVV symptom scores are displayed in Tab. 1. In general, infested beets did not show the ordinary beet shape, but developed a very small "T-like" phenotype. Frequently, leaves displayed typical yellow veins and brownish vascular system. The scoring of all genotypes in various soils was closely connected to beet weight. All soils caused severe symptoms on 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. Peculiar, despite fungicide treatment *Rhizoctonia solani* symptoms were identified especially on tap roots of *Rz1+Rz2* and *Rz1* sugar beets cultivated in D soil.

Tab. 1: Beet scoring for BNYVV symptoms [discolouration of the tap root, dark brownish hair roots, brown vascular, leaf symptoms (yellow veins)] on fresh harvested sugar beets cultivars

Soil	*Rz1+Rz2*	*Rz1*	*susceptible*
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**

Quantitative BNYVV contents measured by ELISA

To measure the absolute BNYVV content in infested sugar beet hair roots as an attribute to resistance (Giunchedi et al., 1985 & 1987; Bürcky & Büttner, 1985, Büttner & Bürcky, 1990) a quantitative BNYVV ELISA was conducted. Results from quantitative ELISA (Fig. 3) were negatively correlated to sugar beet weight. In all naturally infested soils, hair roots from susceptible

cultivar displayed symptoms of severe BNYVV infection, root weight reduction and also high virus contents. Comparing all soils analysed, particularly P produced the highest averaged BNYVV-content (105.25 ng ml^{-1}) in lateral roots of the susceptible cultivar. In R-, GG-, IV- and MN-soils BNYVV susceptible beets exhibited mean virus contents between 40.31 ng ml^{-1} (IV) and 74.48 ng ml^{-1} (R), whereas the same cultivar in D- soil displayed significant lower average BNYVV content (26.85 ng ml^{-1}). As observed in tap root weight analysis, for the *Rz1* containing sugar beet cultivar, the virus content correlated strongly negative, since only beets grown in D-, IV-, MN- and P- soils (46.99 ng ml^{-1} MN to 112.43 ng ml^{-1} IV in average) were heavily infected. In contrast *Rz1* plants grown in R- (mean virus content of 0.01 ng ml^{-1}) and GG-type (in average 0.04 ng ml^{-1}) soil displayed only negligible BNYVV concentrations. Regarding the *Rz1+Rz2* genotype virus content plants in all infected soils displayed significantly lower virus contents than both *Rz1* or susceptible genotypes. In detail, soil from R and GG (both 0 ng ml^{-1}) strongly differed compared to mean values of IV (12.69 ng ml^{-1}), D (6.15 ng ml^{-1}) and MN (4.69 ng ml^{-1}). The ELISA absorbance for P can be neglected, as it displays a virus content of only 0.03 ng ml^{-1}. In resistance tests with four weeks cultivation time in D-, IV-, MN- and P-soils with an *Rz1+Rz2* genotype BNYVV contents were observed to be 10 to 20 times higher (data not shown). Sterile sand served as control for both ELISA background and contamination between soils and as anticipated, no virus could be detected in plants of all three different genotypes (Tab. 1 and Fig. 3).

Fig. 3

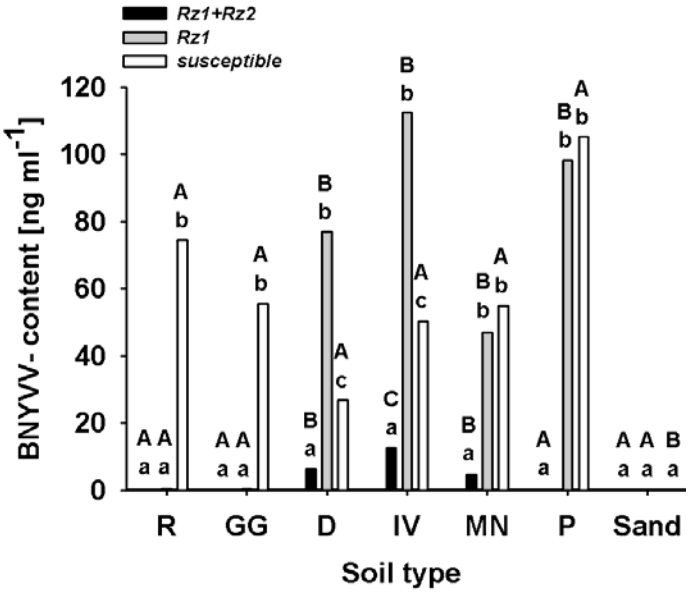

Fig. 3: Average BNYVV content [ng ml^{-1}] after quantitative ELISA from different sugar beet cultivars (black: *Rz1+Rz2*; grey: *Rz1* and white: *susceptible*) after 12 weeks greenhouse cultivation in infested Rhizomania soils from R (Rovigo/Italy), GG (Groß Gerau/Germany), D (Diamiel/Spain), MN (Minnesota/USA), IV (Imperial Valley/USA), P (Pithiviers/France) and an autoclaved sand control. Means within the same soil type (minuscule) and means within genotypes (capital) with a letter in common are not significantly different at the 5% level.

Identification of different BNYVV isolates and sequence analysis

The identification of BNYVV type was carried out by BNYVV-P25 RT-PCR amplification followed by sequencing. Hair-roots from sugar beets grown in R, GG, D, IV, MN and P soils contained BNYVV that was detected by P25 RT-PCR (Fig. 4). In the sterile sand (S) control as well as the water control (NC) no BNYVV-P25 amplification and thus no band could be shown. The positive control (PC; a P25 gene containing plasmid) and the infested soil samples exhibited a single band at 657 bp. The P25 PCR fragments were sequenced and the amino acid (aa) tetrad position 67-70 was determined (Tab. 2). Sequences of R-soil derived P25 displayed AHHG composition, P25 from GG was sequenced as AYHR, beets cultivated in D showed a mixed infection with P25 tetrad ACHG and VCHG. The US-soils IV (VLHG) and MN (VCHG) exclusively possessed valine on position 67 and P differed from all other types displaying an SYHG tetrad. Furthermore in P-soil samples the additional RNA-5 was detected using P26 open reading frame specific RT-PCR (data not shown).

Fig. 4

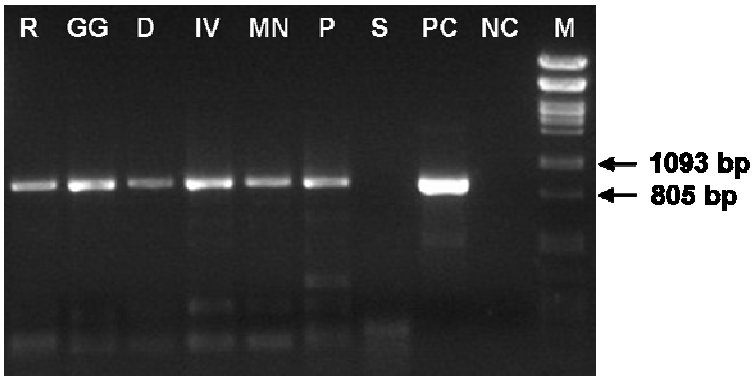

Fig. 4: Detection of a 657 bp BNYVV RNA3 RT-PCR fragment from beets cultivated in R (Rovigo/Italy), GG (Groß Gerau/Germany), D (Daimiel/Spain), MN (Minnesota/USA), IV (Imperial Valley/USA), P (Pithiviers/France) and an autoclaved sand control (S) separated on agarose gel. An RNA3 cDNA containing plasmid served as positive control (PC) and a water control as negative control (NC).

Tab. 2: Sequenced aa67-70 motif on RNA3 encoded P25

BNYVV source	***Nucletide position number***			
	67	***68***	***69***	***70***
R	A	H	H	G
GG	A	Y	H	R
D	A/V	C	H	G
IV	V	L	H	G
MN	V	C	H	G
P	S	Y	H	G

Relative quantification of Polymyxa betae via Polymyxa specific GST ELISA

An assay to determine the potential to multiply and produce secondary zoospores was conducted, as there was evidence from previous studies that enhanced viruliferous vector multiplication and BNYVV transmission might be involved in resistance breaking (Scholten et al., 1994). Lateral roots of test plants were used to determine the concentration of *P. betae* zoospores by means of Polymyxa specific GST ELISA. In a test for presence and relative quantity of *P. betae* zoospores the ELISA displayed significant differences in *P. betae* progeny potential in different soils analysed (Fig. 4). MN soil samples resulted in the highest *P. betae* concentration (from 1747.44 to 4018.24 GST ml^{-1} root sap). D and P obtained on average five to nine times less *P. betae* GST protein content compared to MN. R and GG contained 16 times less *P. betae* in comparison to MN. A dependence of different genotypes within different soils however was not evident.

Fig. 5

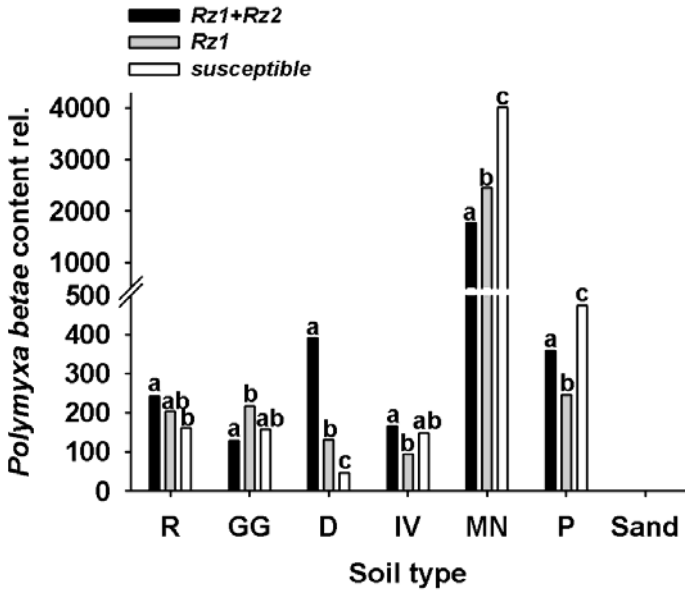

Fig. 5: Average ELISA values of relative *P .betae* protein content from different sugar beet genotypes (black: *Rz1+Rz2;* grey: *Rz1* and white: *susceptible*) after 12 weeks greenhouse cultivation in infested Rhizomania soils from R (Rovigo/Italy), GG (Groß Gerau/Germany), D (Daimiel/Spain), MN (Minnesota/USA), 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.

P. betae variability - Sequence and phylogenetic analysis

All tested soils originated from different regions worldwide. As *P. betae* isolates were derived from the same host, possibly sequence variability might be connected to proximate adaptation to climatic conditions etc. Genomic variability might influence i.e. virus transmission efficiency, the ability to multiply and the ability for long-term survival in soil. Within this study, ITS1+5.8S+ITS2 rDNA

sequences from all *P. betae* isolated (R, GG, D, IV, MN and P) were determined and sequence alignment with rDNA sequences from one English *P. betae* isolate (Ward & Adams, 1998, Acc. no. Y12827), different *P. graminis* ssp. and other plasmodiophorids (*Ligneria* sp. and *Plasmodiophora brassicae*) was carried out. ClustalX alignment resulted in three different main branches which were assigned to group 1-3 (Fig. 6). Group 1 shows *P. betae* isolates which originated from different soils in Europe and the USA. Remarkably, there was a very high homology of *P. betae* for this specific sequence within this group. Only very few single nucleotide exchanges were detected in the ITS1+5.8S+ITS2 region, leading to a very close phylogenetic distance between the different *P. betae* isolates in this study. However, *P. betae* could be distinctly separated from *P. graminis* (group 2). Within group 2, alignment resulted in clear separation of two subgroups (*P. graminis* f.sp. *temperate* & *P. graminis* f.sp. *tropicalis* and *P. graminis* f.sp. *colombiana* & *P. graminis* f.sp. *tepida*) after pairwise comparison. The third group was build by two other plasmodiophorids (*Ligneria* sp. and *P. brassicae*).

Fig. 6

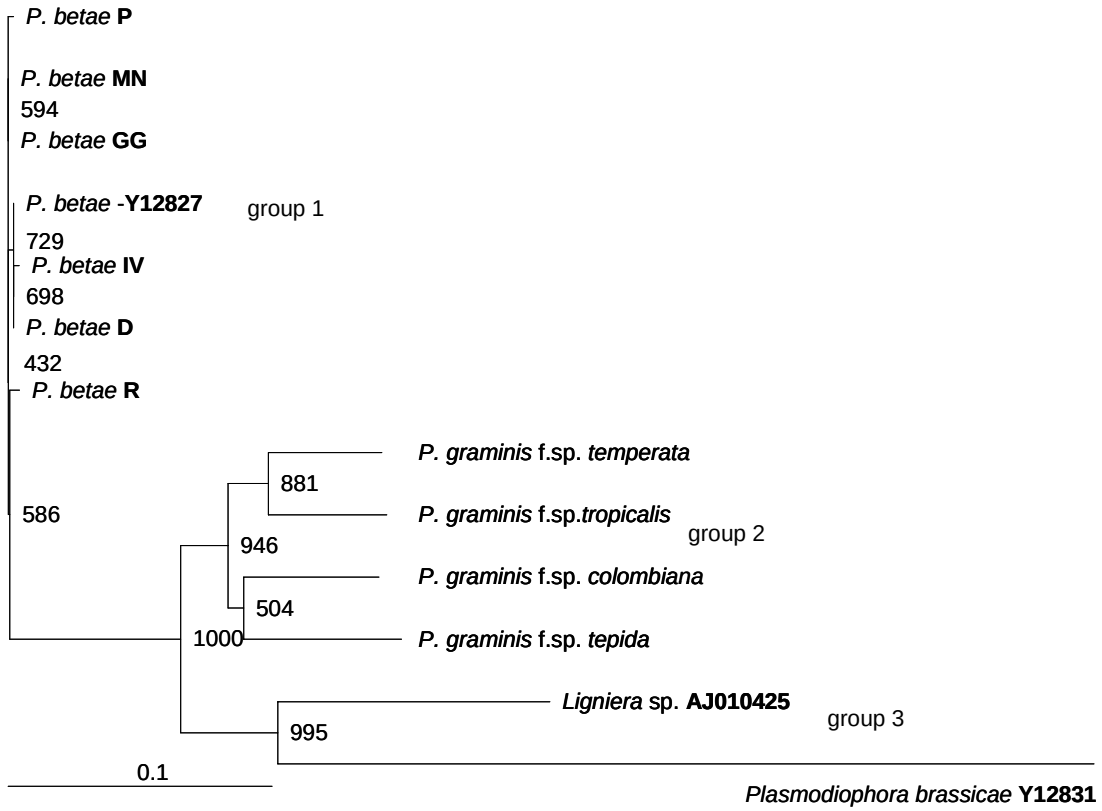

Fig. 6: Phylogenetic analysis of different *Plasmodiophoromycetes* based on rDNA ITS1+5.8S+ITS2 gene. The sequences were aligned and neighbour-joining trees were constructed using the CLUSTAL X program. Boot strap values were calculated from 1000 replicates and are indicated at each node. The scale bar indicates 0.1 % substitution per bp. *P. betae* (GenBank Acc. no Y12827 and isolated from A-, B-, P-, D-, IV- and MN-soil in 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).

Identification of other soil-borne pathogens

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

Discussion

The occurrence of soils containing aggressive BNYVV strains which display the ability to cause reductions in root yield and quality in Rhizomania resistant cultivars have been reported from several sugar beet growing areas throughout the world (Tamada et al., 1996; Heijbroek et al. 1999; Liu et al., 2005; Liu & Lewellen 2007). This study describes the first comparative investigations on resistance breaking abilities of BNYVV infected soils, which contain four RNAs, from European and North American sugar beet growing areas under standardised greenhouse conditions in a resistance test. BNYVV resistance is characterised by reduced virus titres in lateral roots of sugar beets (Bürcky & Büttner, 1985). Thus, relative ELISA absorption value of hair-root sap and absolute quantification of BNYVV structural coat protein was used to test whether resistance breaking occurred. Additionally the cultivation time of identical genotypes was prolonged from 4 weeks (Liu et al., 2005) to 12 weeks in the greenhouse resistance test to observe the influence of virus infection on root development, to determine if the virus titre correlated with the degree of resistance and whether symptomatic root development occurred.

For this study three different genotypes were examined in soils including soils that were known to produce severe symptoms on partial resistant sugar beets. As controls two reference soil samples were applied, a German BNYVV B-type (GG) and an Italian A-type containing soil (R), for which Heijbroek et al. (1999) had already demonstrated minor BNYVV effects on *Rz1* and Rizor cultivars, and a sterile sand control. The root yield of susceptible genotypes differed only marginally between treatments. However, P seems to show stronger inhibition of root development. Heijbroek et al. (1999) reported that GG seems to be least pathogenic; this is also supported by our

study although different genotypes (varieties and lines) were used. The extreme reduction of *Rz1* plant root weight (similar to control plants) in P-soil compared to R is remarkable. Contrary to previous observations this might be explained by the longer growth time (12 weeks instead of 9 weeks) and the different plant genotypes used in our study. Root development of *Rz1* + *Rz2* plants did not reflect differences in BNYVV pathogenicity derived from R-, GG- and P-soil. The differences in beet weight corresponded to the variations in BNYVV contents measured in lateral roots. BNYVV-P produced the highest virus concentration in *Rz1*, comparable to virus titres in the susceptible controls in R and GG (which both did not produce detectable BNYVV infections in *Rz1* plants). The French P-type is phylogenetically classified as an A-type carrying an additional RNA5. In addition to our observations, several studies reported evidence for a synergism of RNA3 encoded P25 pathogenicity factor and RNA5 encoded P26 for the ability to overcome *Rz1* (Tamada et al., 1996; Miyanishi et al., 1999; Heijbroek et al., 1999). However, we did not observe resistance breaking in plants carrying the combination of *Rz1* and *Rz2* after 12 weeks. But finally only pseudo recombinant isolates of different BNYVV-A- and P-type isolates and efficient artificial inoculation techniques of sugar beet roots will help to give the final proof for this hypothesis. Whether stable resistance is solely based on the presence of *Rz2* or due to a quantitative effect of both major genes play a decisive role needs further investigation. Altogether, these results support a possible interaction of RNA5 encoded P26 with the *Rz1* resistance gene.

Regarding the root development and virus multiplication data of *Rz1*+*Rz2* plants grown in D-soil, a reduction of tap root weight and a positive BNYVV ELISA value was demonstrated. No yield reduction but detectable virus contents were also measured in the American soils. Thus, overcoming of resistance seems to be time-dependent, as in resistance tests cultivated for four weeks all BNYVV isolates from D, IV, MN and P were able to induce higher virus contents in lateral sugar beet roots. Although D-, IV- and MN-isolates possess only four RNAs, increased pathogenicity represented by root weight and BNYVV content of infected *Rz1* plants was comparable to the P-treatment. Since all soils tested were used in the same dilution without determining and adjusting inoculum density, we cannot state whether inoculum density has an influence on the resistance breaking abilities of P-type and D-isolate as it was reported previously (Scholten et al., 1994).

The D-soil from the south of Spain, characterised in this study, contains BNYVV-A-type (Schirmer et al., 2005). As 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. D-isolate P25 possesses the tetrad composition VCHG/ACHG. The soils in this study containing resistance breaking isolates from the United States derived from IV and MN

obtained in both cases valine on position 67. However, Liu & 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 partial resistant sugar beets. In this study, the authors characterized resistance breaking abilities in young seedling assays. In a four weeks resistance test we could confirm these data, obtaining high ELISA values for MN and IV, but also for beets grown in European soils like D and P (data not shown). To analyze the recovering abilities of resistant cultivars from BNYVV infestation, we compared the soils after 12 weeks cultivation time. Both American and the Spanish isolates were able to produce detectable ELISA-values in *Rz1* and *Rz1+Rz2* cultivars, respectively under these experimental conditions. Root weight and virus content results obtained with *Rz1* plants strongly supported previously published data, however, in double resistant plants cultivated in IV, MN and D, higher BNYVV levels were detected than in plants grown in R- and GG- soils, but orders of magnitude lower than detected in *Rz1* plants. Virus contents obtained showed a good correlation to the (resistance breaking) influence of both the US-isolates on *Rz1* but not *Rz1+Rz2* cultivars. This might be explained by the different experimental setup used in our study. However, it corresponds to the reported reduction of white sugar yield in *Rz1+Rz2* cultivars grown in the Imperial Valley region (Liu et al., 2005). These observations allow the speculation, that *Rz1+Rz2* could be compromised, once this combination is commonly cultivated in most sugar beet production areas, similar to the resistance breaks in various soils with *Rz1* genotypes. The data obtained in our study give evidence that further multiplication of BNYVV is still occurring in sugar beets possessing both resistance genes, possibly increasing the accumulation of virus potential in soil.

For successful BNYVV-transmission a sufficient number of viruliferous *P. betae* zoospores in soil is a prerequisite (Asher et al., 2003). The vectors ability to multiply in sugar beet roots plays a significant role regarding a possible resistance break (Scholten et al., 1994). Different vector concentrations in soil were used, thus it was essential to compare *P. betae* concentration and propagation potential. Obviously, cultivars in R, GG and IV did not differentiate 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 similarly in susceptible and partial resistant cultivars. *P. betae* augmentation was different in susceptible and partial resistant genotypes grown in D, MN and P, whereas no generalisation concerning the cultivar was shown. However, the noticeable high *P. betae* $\text{content}_{\text{rel.}}$ in MN soils (in average 10 times more *P. betae* than in all other measured soils) shows quite effective *P. betae* propagation. 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) cannot be implicated in this soil, since the virus content as described above, was not

extraordinary high. MN soil contained high numbers of zoospores which are either non-viruliferous or have a low ability to transmit BNYVV. The low *P. betae* $content_{rel.}$ in the BNYVV susceptible (only a tenth of all other measurements) and the *Rz*1 cultivar in D soil is impressive. It is suggested that the great deterioration of lateral and tap roots 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).

However, the *P. betae* content in lateral hair roots cannot be the only significant factor for the vectors successful transmission of BNYVV. As consequence, sequencing of ribosomal ITS1, 5.8S gene and ITS2 genomic region of *P. betae* was carried out to see if *Polymyxa* from various origins might show phylogentic diversification.Legrève et al. (2002) showed genomic diversity in *P. graminis* regarding ITS sequences, leading to the proposal of subspecies, but no differences between two geographically distinct *P. betae* isolates (Belgium and Turkey). Differences between *P. graminis* and *P. betae* in the region amplified with *Polymyxa* specific primers were obvious, as well as diversity within *P. graminis* subspecies. Interestingly, all *P. betae* types, although originating from different places worldwide, hardly differed in the sequence analysed, not allowing phylogenetic differentiation. However, this does not exclude differences in the ability to multiply, take up and transmit BNYVV and therefore more detailed genetic analysis is necessary to unravel variability in these virus vector properties.

Several beets displaying discolouration were tested for additional infestation by other soil born fungi since additional soil born pathogens must be considered to influence sugar beet yield and BNYVV content. Beets grown in D-soil contained secondary soil-borne pathogens that may play an important role concerning compromised performance of partial resistant cultivars. Sugar beets in this soil showed extremely diminished growth throughout the cultivation period. Typical black discoloration of hypocotyls and early damping-off are symptoms for infestation with *Pythium, Fusarium* and *Rhizoctonia solani*. Seed treatment and usage of Tachigaren seven days after planting did not prevent the fungal damage. Especially, severe infections with *R. solani* and *Pythium sp.* in synergism with BNYVV independent of the genotype could lead to extreme weight reduction and increased virus titres, as long as the beet is not completely destroyed as demonstrated in D. A possible explanation for resistance-breaking phenotypes in highly infected soils like D could be a preliminary infection by above mentioned pathogens. Proximate, viruliferous *P. betae* may affect beets which are not able to genetically defend vector and virus multiplication in lateral roots due to heavy primary damage. Remarkably, secondary soil-borne pathogens (*Pythium* and *Fusarium*) were also detected in the other soils, but did not induce these severe symptoms observed in D. Presumably, the density of other pathogens and BNYVV is lower, and only marginal discolouration on beets in all soils other than D may be an explanation for the concentration of

infectious pathogenicity units in the soil. This results in soil-born pathogen infections beside BNYVV in all soils, but apparently sugar beets recover or use a genetic defence mechanism against the penetrating organisms, whereas in D-soil the pathogenicity pressure is so high, that resistance against BNYVV is compromised.

Taken together this study shows that i) *Rz1* resistance in sugar beet is compromised in soils from Spain, the USA and Pithiviers ii) the vector *P. betae* from soils of different regions worldwide did not differentiate in the ITS1+5.8S+ITS2 rDNA region that would indicate evolutionary development after geographic distribution of Rhizomania to various sugar beet productions areas. iii) the vector concentration in soil shows a strong variation regarding different soil origins iv) other soil-borne fungi might play an important role regarding disease severity of BNYVV, but experimental evidence is lacking, yet.

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MANUSCRIPT II

Submitted to Plant Disease

Breaking of beet necrotic yellow vein virus resistance in soils 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. For almost three decades, sugar beet yield was kept more or less stable by cultivating partial resistant sugar beet varieties. These sugar beet cultivars contain single (*Rz1*) or double (e.g. *Rz1+Rz2*) resistance genes. Recently, resistance breaks in *Rz1* and *Rz1+Rz2* genotypes, respectively, have been observed in North America and in Europe. An approach to clarify whether breaking of resistance is dependent on inoculum density was done by estimating inoculum concentration of BNYVV and *Polymyxa betae* using the most probable number method (MPN) for soils from Europe and North America. Since the MPN resulted in highly significant differences regarding the virus concentration in various soils, the same MPN was conducted with *Rz1+Rz2* partial resistant sugar beets. Similarly, the concentration of *P. betae* developing zoospores in roots was determined via specific detection of fungal glutathione-S-transferase in serial diluted soils. Experiments with normalised virus inoculum added to sterile soil showed that resistance breaking does not correlate with virus concentration in soil. Apparently, BNYVV isolates from specific regions in Spain, the USA and France seem to be able to overcome *Rz1* derived resistance in sugar beet by specific host interactions.

Introduction

Economically, Rhizomania is one of the most important sugar beet diseases world-wide. In 1958 beet necrotic yellow vein virus (BNYVV) - commonly known as Rhizomania - was identified in Italy (8) to be the causal agent for severe yield and sucrose reductions in sugar beet. Until today the disease has spread to all important sugar beet growing areas in Asia, North America and Europe (3, 27, 34, 46). BNYVV is transmitted by the obligate biotrophic plasmodiophoromycete *Polymyxa betae* Keskin (1, 16, 45, 52). Yield reduction and Rhizomania symptoms in susceptible cultivars are dependent on the presence and density of viruliferous *P. betae* (48). The vector itself hardly affects sugar beet growth (40). Along with *P. betae* and hence BNYVV density in soil, factors like soil moisture and soil temperature also play an important role in respect to Rhizomania symptom severity (48).

To date, Rhizomania is controlled by growing sugar beet cultivars which contain partial resistance. Meanwhile, the Holly source that contains an important major dominant gene named *Rz1* is the main BNYVV resistance gene in commercially used varieties (4, 28, 36, 43). Additionally, wild beet accessions like *Beta vulgaris* ssp. *maritima* WB41 and WB42 served as sources for additional resistance genes *Rz2* and *Rz3*, respectively (10, 28, 42, 44, 51). Thus, marketable Rhizomania resistant sugar beet varieties contain *Rz1* or a combination of two resistance genes (*Rz1*+*Rz2*) to improve resistance stability through their additive effects. Nevertheless, all known major resistant genes against the virus, either on their own or in combination, provide only partial resistance and are unable to prevent BNYVV infection entirely. Usually they possess the ability to reduce the virus replication in hair roots and inhibit virus spread to the tap root. Thus, the inoculum potential in soils apparently increases continuously.

Molecular analysis of the BNYVV genome resulted in the identification of three major pathotypes: BNYVV-A, -B, and -P, whereas the sequence of A and P within their types display higher variation than those of different B-type isolates (18, 23, 25, 41, 38). The A-type occurs mainly in southern Europe, Benelux, Asia and the USA, whereas the BNYVV-B-type is mainly found in Germany, the upper Rhine valley in France and Switzerland. Koenig et al. (21) initially described the European BNYVV-P-type, which occurs only in a small region near Pithiviers in France and recently at two sites in the UK (11, 50). BNYVV-P is the only European pathotype that contains an additional RNA5, whereas the other two BNYVV-types (A and B) possess only four RNA segments (20, 37). Compared to BNYVV-A and –B, BNYVV-P isolates are characterised by their rapid spread in plants due to the additional RNA5 encoding a second pathogenicity factor (12, 47).

The current methods to detect viruliferous *P. betae* in soil are quite limited. Either bait plant bioassays using soil dilutions are applied to estimate the most probable numbers (MPN) of infective propagules (48) or polymerase chain reaction (PCR) is used to estimate *P. betae* content in plant material and soil (17, 31, 32, 33, 49). In soil from The Netherlands Tuitert (48) estimated that 10-15% of the root infecting *P. betae* population is viruliferous but experiments to describe the inoculum concentration in soils with resistance breaking abilities are still lacking. Same applies for trials to test different inoculum densities.

The objective of this work was to examine *P. betae* and BNYVV densities in soils from Italy, France, Germany, Spain and the USA by MPN and to determine the percentage of viruliferous *P. betae.* In the experiments described here soils that were known to break *Rz1* resistance displayed high MPN values. However, subsequent resistance tests with normalised virus inoculum showed that the inoculum density is not responsible for overcoming of resistance, although the MPN resulted in highly variable virus and vector concentrations in soils.

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 most probable number (MPN) test as described by Tuitert (48) was used in a modified form. The dilution ratio for the serial soil dilution was reduced from 10 to 5. Moreover, the number of dilution steps was raised from 5 to 6 for a better coverage of different inoculum densities.

In the experiments described here, 10 plants were planted into single pots to serve as independent repetitions for each dilution step. Soil samples were air dried and sieved over 2 mm sieves. Autoclaved sand (coarse sized 1-2 mm) served as control. Each soil was thoroughly mixed with dried sterile sand in 40 l plastic bags, starting with the highest concentration (5^{-1}) down to the lowest concentration (5^{-6}). Per test plant 300g of mixed soil of each dilution was filled in plastic folding boxes (sized 4 cm x 4 cm x 16 cm). The MPN calculation was conducted using an MPN calculator, based on maximum likelihood equations following the MPN calculations of Hurley and Roscoe (13). This equation was used 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 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), France (Pithiviers; P) were chosen for serial dilution experiments. The occurrence of the following BNYVV-types was described earlier in the different soils and confirmed in our studies by sequencing of the RNA3-PCR products from plants cultivated in described soils (*data not shown*): BNYVV A-types in R, D, MN and IV soils (12, 39, 41), the B-type in GG soil and the P-type in P soil (12).

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;* (30)] were applied. In experiments using an adjusted BNYVV inoculum a third single-resistant variety [*Rz1rz1,* referred to as *Rz1*; (30)] was included.

Bioassay

Seven days old seedlings, which had been germinated in sterile silica sand, were transplanted into fully water saturated soil. Seven days after planting, sugar beets were sprayed with fungicides (0.2 g/l Tachigaren 70 W.P., Sumitoma, 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 16 h of assimilation light.

BNYVV DAS-ELISA

After 4 weeks of greenhouse cultivation, sugar beet plants were harvested individually. Sand and soil were thoroughly removed by washing with running tap water. Lateral roots were separated from the tap root and dried with paper towels. Leaves and the hypocotyls were discarded.

The quantitative DAS-ELISA was conducted following the method described by Gidner et al. (10), with slight modifications. Plant sap from lateral roots of each plant was gained via a Pollähne leaf juice press (MEKU GmbH) and PVP-Tween-buffer (24) at a ratio of 1:15 (root weight/buffer in g ml^{-1}). Each ELISA plate (Nunc A/S, Roskilde Denmark) besides blanks and buffer-controls contained the following sample allocation: 2 samples of healthy and infected plant sap, respectively, and an additional dilution series from a *Chenopodium quinoa* BNYVV purification

prepared following the method described by Koenig et al. (22). The dilution series, used to develop a standard curve, was based on 4000, 2000, 1000, 500, …, 1,95 ng virus protein per ml buffer, 0 ng ml^{-1} with reference to the mean of tested healthy controls plus three times the standard error. To keep comparability between subsequent tests, aliquots of the standard (4000 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 (405nm) of the colour reaction was measured after one hour incubation at 37°C using a Titertek Multiskan Plus photometer (Magarete Malar, Nauheim, Germany). Samples were considered to be infected if the absorption resulted positive (BNYVV content > 0) after subtracting the absorption of healthy controls, plus three times standard deviation, plus blanks.

Quantification of Polymyxa betae zoospores (by quantitative ELISA)

The quantification of *P. betae* by TAS-ELISA via specific detection of fungal glutathione-S-transferase (GST) was carried out according to Kingsnorth et al. (17) with some modifications. The same lateral root samples used for BNYVV quantification were applied in the *P. betae* ELISA. All incubation steps of the *P. betae* TAS-ELISA were performed at 37°C for 1 h. As alkaline phosphatase tagged antibody, the anti-rabbit antibody (IgG whole molecule, Sigma-Aldrich, Munich, Germany) was used. To quantify *P. betae* expressed GST, a standard curve with purified GST [described in Kingsnorth et al. (17) and kindly supplied by Broom´s Barn research station, UK] in a dilution series from 1/2048, 1/1024, 1/512, … , to 1 was analyzed parallel on the ELISA plates. The highest concentration (abs_{405nm} 1.2, calibration 1/2048) corresponded to a fungus ratio of $GSTconc._{log}3000$, the calibration 1 (abs_{405nm} 0.007) displayed a $GSTconc._{log}0$, respectively.

Inoculum preparation (Resistance tests with standardized inoculum)

Lateral sugar beet roots from susceptible sugar beet cultivars, previously cultivated for 12 weeks in R, GG, D, IV, MN and P soils and in sterile sand, were used to standardize the BNYVV inoculum. For that, beets were harvested, washed with tap water and leaves were discarded. Lateral roots were separated from the tap root, collected and air dried at room temperature for three days. Dried lateral roots were cut into small pieces (max. 2 mm) and thoroughly mixed. An aliquot was used for determining the BNYVV concentration by quantitative DAS-ELISA. After quantification of the virus content in each homogenized lateral root bulk from beets grown in R, GG, D, IV, MN and P soils, the samples were diluted in damped soil to a final concentration of 70 ng BNYVV per 1 kg sterilized damped soil [sand: clay mixture (1:2)]. Plants which were harvested after four and eight

weeks were potted into 1 kg of soil and plants which were harvested after 12 weeks into 2.5 kg of soil per pot. Homogeneous dispersal of the inoculum was achieved by equally pouring a 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 single sugar beet plants served at harvest as repetitions.

Bioassay with lateral root inoculum and BNYVV analysis

The bioassay was conducted corresponding to the MPN tests. After 4, 8 and 12 weeks of greenhouse cultivation sugar beet plants were harvested individually. Soil and inoculum were thoroughly removed from tap and lateral roots by washing with running tap water. Plants were subsequently dried with paper towels and the whole variant was scored from 1 (no infection) to 9 (fully infested tap roots) for BNYVV and occurrence of *Rhizoctonia solani* (*data not shown*) symptoms. After scoring, lateral roots were separated from the beet body, leaves and hypocotyls discarded and the BNYVV concentration determined by quantitative DAS-ELISA as described above.

Tissue print immuno assay

The systemic spread within sugar beet tap roots was analysed using Tissue Print Immuno Assay (TPIA). Longitudinal sections of 12 week old sugar beet tap roots from the resistance test with standardized inoculum were firmly pressed on positive charged nylon membrane (Whatman, UK). TPIA was carried out exactly as described by Kaufmann et al. (15).

Data analyses

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 (Anonymous, 2007) followed until all data displayed a normal distribution. The ANOVA was conducted with transformed data using the PROC MIXED procedure. All statistically analysed data of this study are presented in the reverted transformed form.

Results and Discussion

For final proof whether resistance breaks depend on BNYVV inoculum concentration experiments were carried out using different BNYVV origins known to overcome resistance. The BNYVV inoculum density and the performance of various sugar beet genotypes in presence of these

BNYVV isolates were determined. Afterwards the virus concentration was equalised and served as inoculum for the infestation of sterile soil. A time series harvest was conducted within this test under standardized inoculum densities to observe the temporal virus propagation and systemic spread in sugar beet. The data obtained by identical analyses of all sugar beet cultivars within different soil dilutions and inoculum densities were comparable and explain the high variability and diverse pathogenicity.

BNYVV inoculum density in resistance breaking soils and virus content in infected susceptible plants

After four weeks greenhouse cultivation, BNYVV susceptible sugar beet plants displayed obvious differences in phenotype and strength of BNYVV symptoms, depending on soil origin and serial dilution. Due to these severe infections with BNYVV or other soil-borne pathogens (*data not shown*) and the resulting plant death, the number of test plants in some soil vs. dilution variants was reduced to 9. To determine the number of infected plants in each treatment, the virus content was assayed using quantitative ELISA to detect in addition to MPN determination correlations of inoculum concentration with the symptom severity observed in the different soil dilution steps.

Lateral root samples were evaluated using ELISA as follows: The calculated MPN-values displayed in Tab. 1 indicate great variations of the BNYVV concentration in different soil origins analysed. German GG-soil showed the lowest inoculum concentration of estimated 11 infective units per g soil (iu g^{-1}), followed by the MN-soil and Italian R-type soil showing 10 to 11 orders of magnitude higher MPNs. In BNYVV-P and -IV soils 30-50 times higher inoculum density was measured. Remarkably, D-soil contained a BNYVV concentration that was 520 orders of magnitude larger than the one detected in GG-soil. When Tuitert (1990) compared different MPN calculation methods in one Dutch soil containing unknown BNYVV-type the results differed between 40-100 iu g^{-1}. Although, different MPN tests are thought to be incomparable due to different calculation methods (48), 40-100 iu g^{-1} represent a BNYVV density comparable to GG, R and MN in our experiments. Similar to Tuitert (48), our trial proved that MPN values up to approximately 100 iu g^{-1} are sufficient to infect susceptible sugar beet plants in up to 5^{-4} diluted soil (R and MN). As MPN determination revealed soils with similar MPN values (i.e. MN and R) but differences in previous reported resistance breaking abilities (29), pathogenicity does not seem to correlate well to inoculum concentration. To our knowledge, this is the first comparison of BNYVV content of different standard and resistance breaking soils under standardized conditions.

Tab. 1: BNYVV concentration in soils from different geographic origins measured with a susceptible sugar beet genotype and determined by DAS-ELISA after four weeks greenhouse cultivation time to calculate the MPN by means of number of infected plants / number of harvested plants shown for each soil variant in different dilution steps.

Dilution ratio = 5	*Genotype: susceptible sugar beet line (rz1rz1)*					
	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 soil	110 a***	11 b	5200 c	510 d	98 a	340 d

* Number of infected sugar beets

** Number of single harvested sugar beets

*** Means with the same letter (MPN/g soil) are not significantly different (LSD 0.05)

In this experiment applying a susceptible line, a high variation of virus concentration among almost all soils was shown which did not correlate to the serial dilution of the soil (Fig. 1). Despite big differences concerning the inoculum density in different soils analysed with a susceptible sugar beet line, results of BNYVV-concentration in relation to soil-origin demonstrated in Fig. 1 show that only P-type soil produced a higher virus content in the lowest soil dilution which might be connected to the additional pathogenicity factor (P26) on RNA5. Independent of the virus inoculum density in soil and soil origin, virus contents in lateral sugar beet roots hardly differed (Fig. 1). Remarkably, in dilutions 5^{-1} until 5^{-3} plant virus contents slightly rose, besides in R and P. Thenceforth the BNYVV content declined to some extent until the virus was out-diluted. Only in D-soil an out-dilution was not achieved, although diluting the soil 5^{-6}. This demonstrates the potential of *P. betae* derived from different soils to multiply and produce similar numbers of secondary infections during the 4 weeks cultivation period. Nevertheless, there was no correlation in the reduction of the average virus content in relation to virus content in soil after the serial dilutions as shown in Tab. 1. An expected correlation between the calculated MPN (Tab. 1) and the high virus contents in lateral roots (Fig. 1) could not be demonstrated.

Fig. 1

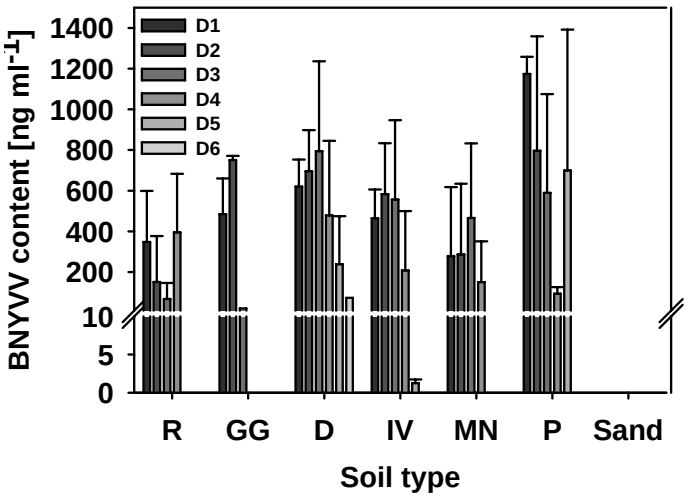

Fig. 1: Means of BNYVV content [ng ml^{-1}] in lateral roots of infected susceptible sugar beet cultivars as determined by quantitative DAS-ELISA. Plants were grown for four weeks in the greenhouse in six dilutions (D1 = 5^{-1}; D2 = 5^{-2}; D3 = 5^{-3}; D4 = 5^{-4}; D5 = 5^{-5}; D6 = 5^{-6}) of soils 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.

BNYVV resistance in sugar beet is a quantitative trait reducing the virus accumulation [reviewed in Rush (40)]. Therefore, the non-infected sugar beet plants were excluded from the evaluation of all soil*dilution variants to estimate the average BNYVV-content per variation and repetition (Fig. 1). Since data show mean values (if more then one beet are infected) the standard variation is included in the figure.

Determination of BNYVV inoculum density in different soils able to overcome Rz1 + Rz2 resistance

A repetition of the MPN-assay applying an *Rz1+Rz2* cultivar (Tab. 2 and Fig. 2) was carried out to investigate if various BNYVV isolates are able to overcome resistance as described by Liu et al. (30) and if the serial dilution might affect the BNYVV content in plants. It was assumed, if overcoming of resistance is depending on BNYVV concentration, the occurrence of infected plants would cease at a dilution step far prior to the out dilution point observed in the MPN with susceptible genotype. However, the results obtained did not give strong evidence for a positive correlation of virus concentration in soil to the ability to produce elevated BNYVV levels in *Rz1+Rz2* plants.

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

Dilution ratio = 5	*Genotype: BNYVV-double resistant variety Rz1+Rz2*					
	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. ovc. Rz***	4,5 a****	0,8 a	20 b	8 ab	13 b	9,4 ab

* Number of infected sugar beets

** Number of single harvested sugar beets

*** Number of BNYVV isolates overcoming resistance

**** Means with the same letter are not significantly different (LSD 0.05)

Fig. 2

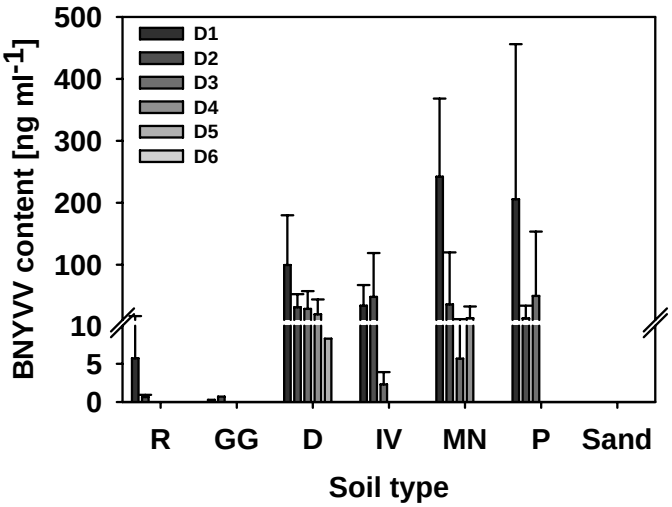

Fig. 2: Means of BNYVV content [ng ml^{-1}] in lateral roots of infected *Rz1+Rz2* partial resistant cultivars determined by quantitative DAS-ELISA. Plants were grown for four weeks in the greenhouse in six dilutions (D1 = 5^{-1}; D2 = 5^{-2}; D3 = 5^{-3}; D4 = 5^{-4}; D5 = 5^{-5}; D6 = 5^{-6}) of soils 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.

It was expected that in addition to the number of infected plants the virus content in lateral roots of *Rz1+Rz2* sugar beets should be significantly reduced in higher soil dilutions. The detailed results, including the number of plants infected in various dilution treatments, are displayed in Tab. 2. Soils

with previously reported resistance-breaking abilities were shown to infect plants at much higher dilutions than standard BNYVV containing soils like R and GG (Tab. 2). The data indicate that even in the highest concentration of infested soil (dilution ratio 5^{-1}) R- and GG-soil were unable to produce infections in *Rz1+Rz2* plants with a significant virus titre (between 0.3 and 5 ng ml^{-1}, respectively) (Fig. 2). In contrast, D-, IV-, MN- and P-type soil produced much higher BNYVV contents in lateral roots (99, 33, 242 and 205 ng ml^{-1}, respectively), which are comparable to virus titres in susceptible plants grown in R- or GG-type soil. Different soils produced significant virus contents in *Rz1+Rz2* sugar beet plants even at higher dilutions, although virus concentration tended to result in a BNYVV decline. D-, IV- and MN-type soils were still able to infect 3 out of 10 double resistant plants at a dilution of 5^{-4}, whereas D-type soil even infected one plant in soil diluted 5^{-5}. In P-soil 4/10 plants were tested positive for BNYVV infection in soil diluted 5^{-3}. Calculating with the difference between MPN of susceptible sugar beets (MPN_{sus} Tab. 1) and BNYVV units per g soil overcoming resistance in the MPN with the *Rz1+Rz2* genotype (MPN_{diff} Tab. 2) and thereinafter dividing the MPN_{sus} by the MPN_{diff} it is possible to calculate the concentration of each BNYVV isolate that is necessary to cause infections in *Rz1+Rz2* double resistant plants. Thus, D-soil still infects *Rz1+Rz2* plants at a concentration of about 8.3 BNYVV iu g^{-1} soil diluted 1/3125, IV-soil at 0.8 iu g^{-1} and MN-soil at 0.2 iu g^{-1} in soil diluted 5^{-4}. In P-soil *Rz1+Rz2* plants were still tested positive at 2.7 iu g^{-1} in soil diluted 5^{-3}. Assigning this calculation to the standard soil-types, R-soil was able to infect *Rz1+Rz2* plants at a density of 4.4 iu g^{-1} and GG-soil at 0.44 iu g^{-1} but only in 5^{-1} diluted soil, despite no relevant virus levels were produced. Although, MN-soil displayed a similar BNYVV density in the susceptible test than R-soil MN-derived BNYVV was able to overcome resistance and to successfully infect *Rz1+Rz2* plants. The same applies for the results of IV and P. Both displaying higher MPNs than MN and R but the isolate was still able to infect *Rz1+Rz2* resistant plants in soils diluted down to 5^{-3} as well as 5^{-4}. Even if D shows an immense MPN the inoculum density can be excluded to be the only factor causing resistance breaks as *Rz1+Rz2* cultivars are still infested in 5^{-5} diluted soil. It can be assumed that inoculum concentration does not play a significant role for infection of more aggressive soils. This is also correlating to the average virus content observed in the MPN with susceptible cultivars.

Determination of inoculum density and percentage viruliferous Polymyxa betae in different resistance breaking soils

As BNYVV inoculum density displayed this extreme variation among different analysed soil samples, a verification of data concerning a possible correlation between the concentration of virus and its vector *P. betae* in the soil was necessary. Tuitert (48) estimated the percentage of viruliferous *P. betae* in soil to be 10–15 %. Therefore, the hair-root saps used for DAS-ELISA to

detect BNYVV were additionally applied for TAS-ELISA based zoospore detection and quantification using Polymyxa GST specific antibodies. The quantitative *P. betae* TAS-ELISA allowed a determination of the *P. betae* multiplication rate. Initial comparisons of *P. betae* zoospore densities in BNYVV susceptible and *Rz1+Rz2* plants did not display significant differences (data not shown). This was in agreement with previous observations (3). Although the genetically present BNYVV resistance in sugar beet genotypes did not influence the *P. betae* infection, samples were taken from *Rz1+Rz2* plant roots only, as they suffered less under BNYVV infestation and displayed a similar root system phenotype throughout the experiment.

Tab. 3: *P. betae* concentration in soils from different origins estimated by GST specific TAS-ELISA based detection of zoospores from hair roots of an *Rz1+Rz2* sugar beet cultivar

Dilution ratio = 5	*Genotype: Rz1+Rz2*		*Polymyxa betae*			
			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 soil	180 a****	67 b	8900 c	840 d	3300 e	960 d
% vir. *P. betae****	61%	16%	58%	61%	3%	35%

* 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)

Regarding the obtained TAS-ELISA results standard R- and GG-soil were the only soils which fulfilled the expectations with respect to an out-dilution of *P. betae* within the serial dilution. These observations are summarised in the *P. betae* MPN (Tab. 3). Very low MPN of infectious *P. betae* units were demonstrated for the standard soils (R and GG). In contrast, D-soil possessed very good conditions for *P. betae* multiplication, also in highly diluted soils, and displayed by far the uppermost MPN (8900 iu g^{-1}), almost 50 times more than the standard Italian soil (180 iu g^{-1}) and even 130 times more than the less aggressive German soil (67 iu g^{-1}). MN-soil possessed 2.7 times less iu g^{-1} than D-soil, which is still the second highest MPN value with 3300 iu g^{-1}. IV- and P-soils contained about a tenth of infectious *P. betae* units compared to D-soil.

Using the identical MPN-equation to determine BNYVV and *P. betae* concentration in the soil it is permitted to quote the proportion between virus and its vector. These proportions reflect the percentage of viruliferous zoospores. Much higher values of viruliferous zoospores could be

determined for R- (61%), D- (58%), IV- (61%) and P-soil (35%) than the previously reported 10–15% (48). MN-soil displayed a very low amount of viruliferous zoospores (3%) compared to all other soils. Thus, the percentage of viruliferous *P. betae* did not correlate to resistance-breaking capabilities in the soil as i.e. MN is known to overcome resistance but showed only low percentage of viruliferous *P. betae* in soil. Successful BNYVV transmission by its vector *P. betae*, what might be connected to overcoming of resistance, may depend on genetically or ecological factors but precise information on vector variability is still lacking. Additionally, 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 influence the vector and inhibit BNYVV-uptake.

Determination of Polymyxy betae reproduction abilities in different soil dilutions

The quantitative TAS-ELISA data obtained from lateral roots were used to estimate the concentration of *P. betae* infectious units in the soil. Absolute *P. betae* concentrations were determined and compared to the different soil dilution treatments. After evaluating data obtained from the quantitative ELISA (Fig. 3) it became obvious that *P. betae* possessed strong cystosory reproduction abilities during the four week experiment independent from the adjusted inoculum density. This is reflected by similar values of Polymyxa quantification in different soil dilution steps, which accounts to a better *P. betae* propagation *in planta* with low prelim inoculum density (14). In contrast, Gerik and Duffus (9) reported that the viruliferous vector multiplied much more efficient when an indigenous avirulent population was already present in soil compared to soil where no *P. betae* population is existent, whereas a virulent population is out-competing aviruliferous *P. betae*. Within the dilution in D-, IV-, MN-, and P-soils the *P. betae* infections increased. In IV- MN- and partly in P – soils *P. betae* started to be out-diluted at the highest dilution (5^{-6}) which is displayed by the vector density resulting to present more likely a bell curve over serial dilution. Remarkable is the quite uniform concentration of *P. betae* over all dilution steps in D-soil. Whereas, *P. betae* does not tend to result in concentration decline, thus D-soil is seemingly a soil that allows *P. betae* to propagate easily (Fig. 3). Comparing results from the highest soil concentration (5^{-1}) from R and GG with IV, MN, and P (Fig. 3) it is obvious that the *P. betae* content in latter soils is much lower than in R and GG. This low vector content in plants may be caused by severe BNYVV infections that inhibit root growth and thus inhibits *P. betae* propagation by suppressing the multiplication of secondary zoospores.

Fig. 3

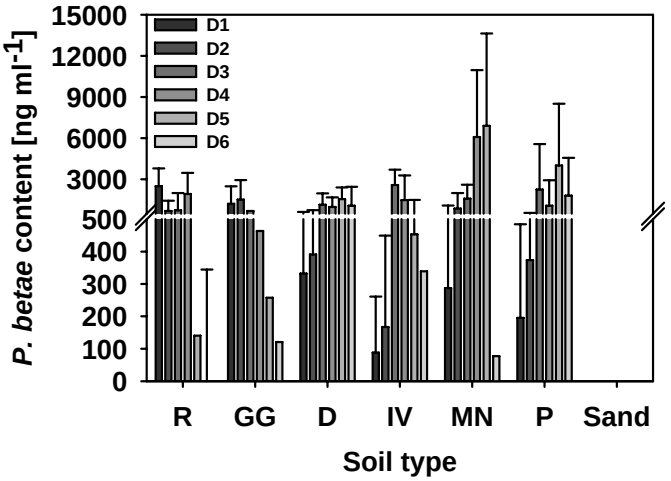

Fig. 3: Means of *Polymyxa betae* content [ng/ml] in lateral roots of infected *Rz1+Rz2* partial resistant cultivars determined by quantitative ELISA. Plants were grown four weeks in the greenhouse in six dilutions (D1 = 5^{-1}; D2 = 5^{-2}; D3 = 5^{-3}; D4 = 5^{-4}; D5 = 5^{-5}; D6 = 5^{-6}) of soils 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.

BNYVV resistance test with adjusted inoculum concentration

It was reported earlier, that breakage of single *Rz1* resistance was observed in naturally infested soils (29, 30, Ayala *personal communication*) or *in vitro* under high viruliferous vector densities (43). In our experiments, the densities of BNYVV and *P. betae* in naturally infested IV-, D-, MN-, and P-soils did not correlate with breaking of *Rz1* or *Rz1+Rz2* resistance. However, the naturally occurring BNYVV isolates in R and GG did not lead to resistance breaks (12). When greenhouse-tests for selection of BNYVV resistance were developed, selection time was shortened compared to field tests to four to six weeks as lateral root virus content of sugar beet seedlings indicate the genotypic resistance potential already at that developmental stage (6, 7). Therefore this growth time was used in most previous studies (e.g. 10, 26, 27, 30, 35, 44). In field resistant tests, however, the virus content in tap roots remains first choice because of decreasing virus concentrations in lateral roots throughout the vegetation period (6).

To consider this knowledge in a test to evaluate if BNYVV content and severe virus symptoms correlate, a resistance test with adjusted inoculum concentration and three harvest dates (4, 8 and 12 weeks) was carried out. A BNYVV inoculum of 70 ng virus kg^{-1} soil was used. This concentration was averaged after determining the BNYVV content by quantitative DAS-ELISAs in resistance tests using different sugar beet genotypes grown in naturally infested R-, GG-, D-, IV-, MN and P-soils (*data not shown*). The four weeks test did not result in significant differences in virus content (*data not shown*) that it was assumed that the encystations of resting *P. betae* spores

from dried root material, used for artificial inoculation, took longer than infestation of viruliferous *P. betae* derived from naturally infested soil, hence, no sufficient infections could be set.

After eight weeks (Fig. 4A) plants of the susceptible cultivar displayed significant virus contents in R-, IV- and MN-soils, indicating strong infestation induced by the different added inoculum sources. Remarkable were the low virus contents in *Rz1* at R and GG corresponding to previous observations of Heijbroek et al. (12), who compared an *Rz1* sugar beet genotype in naturally infested soil from R, GG and P and detected low virus content in R and GG. BNYVV concentration in D- and IV-soil grown susceptible and *Rz1* sugar beets displayed to be high. Surprisingly, the susceptible genotype in D-soil displayed lower BNYVV content than the *Rz1* genotype, what might be related to a totally depauperated and rotten root system induced by the high infection pressure. Apparently the virus multiplication set in soon after the first harvest. Consequently the virus propagated extreme quickly, especially in the susceptible cultivar in IV-soil. IV resulted in the highest BNYVV density in *Rz1+Rz2* (40 ng ml^{-1}) lateral sugar beet roots. Whereas *Rz1+Rz2* in GG did not support virus multiplication (5 ng ml^{-1}) or, at least inhibit any further spread into the lateral roots. Interestingly P did not produce very high virus contents, neither in the susceptible nor in the *Rz1* (both 13 ng ml^{-1}) cultivar, indicating that no infection had occurred at all.

Fig. 4

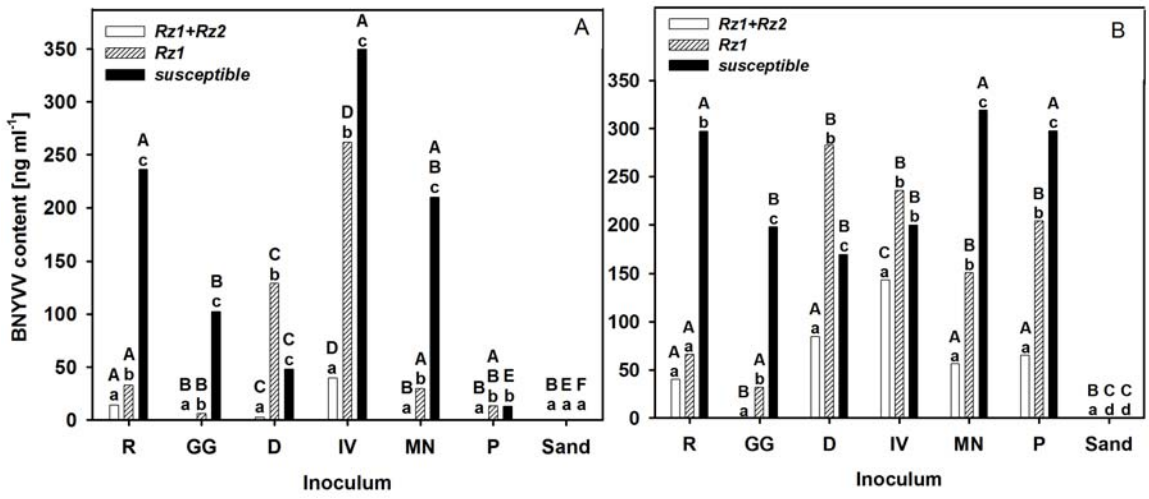

Fig. 4: BNYVV content [ng ml $^{-1}$] in lateral roots in double (*Rz1+Rz2)* and single (*Rz1)* resistant cultivars as well as in a susceptible control after 8 (4A) and 12 (4B) weeks greenhouse cultivation in artificially infested soil with different BNYVV isolates (from R-, GG-, D-, MN-, IV- and P-soil and a sand control). Means within the same soil type (minuscule) and means within genotypes (capital) with a letter in common are not significantly different at the 5% level.

Finally, the harvest and DAS-ELISA after 12 weeks (Fig. 4B) resulted generally in much higher BNYVV concentrations in sugar beet lateral roots than the 8 weeks test. Again, virus contents produced by the BNYVV isolates in different cultivars were significantly distinguishable. Comparing the virus titres in hair roots from P-soil harvested after 8 and 12 weeks of cultivation an immense virus increase was observed in all genotypes, nicely fitting to the above-mentioned observations. Thus, the susceptible genotype achieved a virus content as high as 297 ng ml^{-1}. Additionally, a BNYVV content of 204 ng ml^{-1} in hair roots from P-soil grown *Rz1* sugar beets indicated for resistance breaking. Surprisingly, even *Rz1+Rz2* genotypes showed a high BNYVV content after 12 weeks in hair roots from P-soil (65 ng ml^{-1}), but also in hair roots from beets grown in R-, D-, IV- and MN-soils. Applying these BNYVV isolates it was often possible to significantly distinguish the virus content between the susceptible and *Rz1* cultivars (Fig. 4B minuscule). In some variants (D and IV) the *Rz1* cultivar obtained even higher virus contents than the susceptible genotype (Fig. 4B). This can be explained by severe Rhizomania symptoms severely reduced lateral root growth observed in the susceptible genotype. Similar to observations made after 8 weeks they might have inhibited further virus propagation in totally rotten roots (*data not shown*). Both reference soils (R and GG) displayed the highest BNYVV contents in the susceptible genotype, but in contrast to the data obtained in MPN, detectable virus contents in the both resistant cultivars were observed.

In order to confirm the data obtained in the quantitative ELISA, total plants and sugar beet tap roots were also weighed after harvest (Fig. 5). Noticeable were the high sugar beet weights of all isolates in *Rz1+Rz2* cultivars. Within *Rz1+Rz2* genotype no significant weight differences could be measured between GG-, D-, IV-, MN- and P-soil or sand control grown tap roots. Moreover, the weight of tap roots grown in R-soil was even significantly higher compared to all other variants. In the *Rz1* variants, tap root weights were strongly negatively correlated to BNYVV contents (*data not shown*). The higher the virus titre in lateral roots (Fig. 4B), the more the tap root weights were reduced (Fig. 5). Tap root weight in *Rz1* sugar beet plants did not significantly differ between R-, GG-soil and sand but in D-, IV-, MN- and P-soil grown *Rz1* tap roots, weight was so much reduced, that no significant difference compared to the susceptible genotype could be measured.

Fig. 5

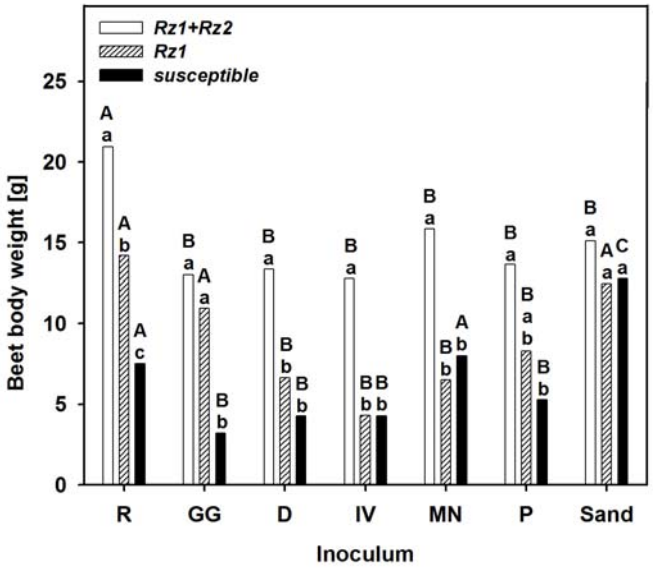

Fig. 5: Tap root weight [g] in double (*Rz1+Rz2)* and single (*Rz1)* resistant cultivars as well as in a susceptible control 12 weeks after greenhouse cultivation in artificially infested soil with different BNYVV isolates (from R-, GG-, D-, MN-, IV-, P-soils and a sand control). Means within the same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

Thus, the tap root weight analysis supported the data achieved in the BNYVV DAS-ELISA and the data allow the conclusion that resistance breaks in resistant genotypes can be displayed also by weighing. Other than published results that showed no significant differences in *Rz1* resistant plants grown in R-, GG- and P-soil (12) the tap root weight of plants grown in P could be differentiated clearly from R and GG after 12 weeks of cultivation in our trials. In order to demonstrate, if virus infection of lateral roots had led to systemic virus spread in sugar beet roots and caused weight reduction in *Rz1* and *Rz1+Rz2* genotypes a TPIA on longitudinal beet sections was carried out (Fig. 6). The developed colour reaction showed systemic spread of BNYVV in susceptible plants grown in all soils. Partial systemic virus infection with unequal distribution was only detectable in *Rz1* resistant plants grown in D-, IV- MN- and P-soil. Tap roots of *Rz1* plants of GG and R treatments displayed no detectable substrate reaction, which nicely correlated to the virus contents of lateral roots. Remarkably, a systemic BNYVV spread in the *Rz1+Rz2* cultivar was only detectable in D- and P-soil grown beet roots. Despite *Rz1+Rz2* lateral roots were significantly infected as shown above, no colour reaction above background was observed in this assay in all plants tested. Despite TPIA detection limit is far beyond quantitative DAS-ELISA (15) and the colour reaction can only be evaluated semi-quantitatively, the TPIA data obtained here allow the conclusion that BNYVV D-, IV-, MN- and P-soil derived isolates in addition to produce elevated virus contents in lateral roots are able to systemically infect *Rz1* plants in this assay. Similar abilities were demonstrated for D- and P-isolate in *Rz1+Rz2* plants.

Fig. 6

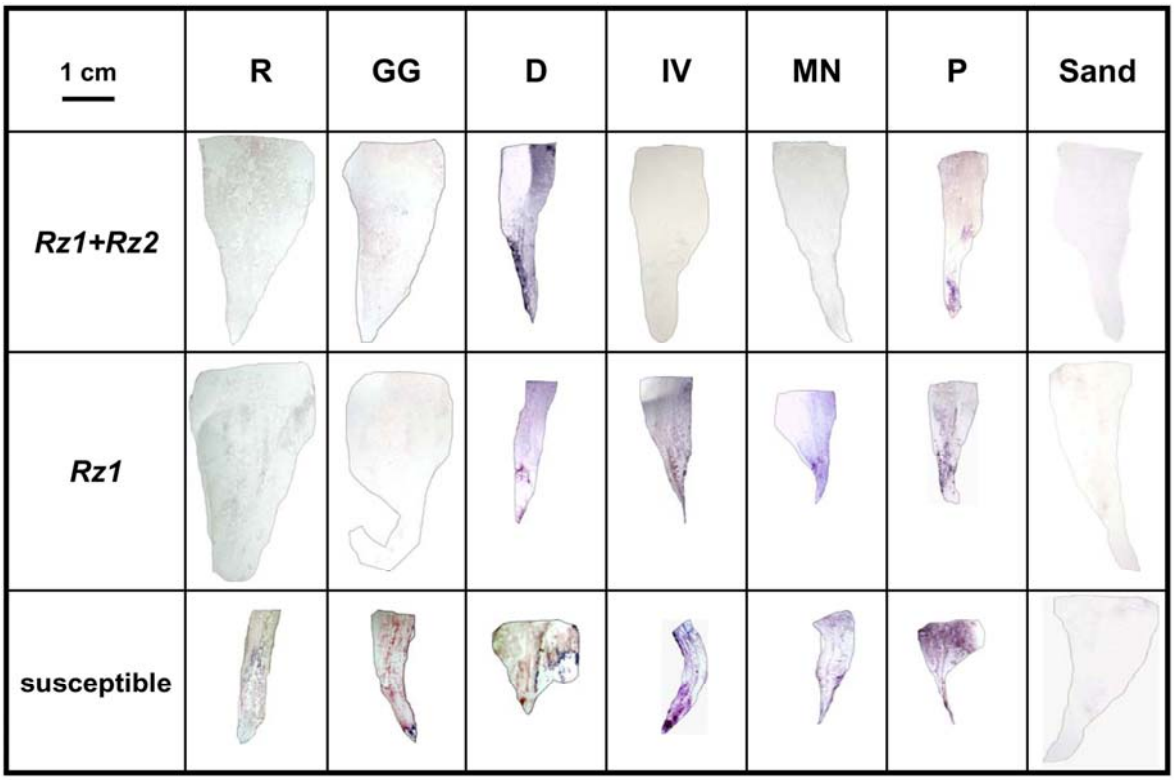

Fig. 6: Detection of systemic BNYVV spread in sugar beet tap roots after TPIA in double (*Rz1+Rz2)* and single (*Rz1)* resistant cultivars as well as in a susceptible control after 12 weeks greenhouse cultivation in artificially infested soil with different BNYVV isolates (R, GG, D, MN, IV, P and a sand control). Dark bluish discoloration shows the virus distribution.

Conclusions

The obtained results give strong evidence that i) there is a high variability in BNYVV content in soils from different origin, ii) most soil samples (D- , IV- and P- but not MN-soil) analysed in this study, which display the ability to produce BNYVV infections under controlled conditions of a certain titre, possess a higher inoculum concentration than soils displaying normal aggressiveness like in R and GG but iii) the resistance overcoming ability is not correlated to the inoculum concentration iv) the *P. betae* concentration itself and the concentration of viruliferous *P. betae* are not clearly correlated to resistance breaking. v) The tap root weight reduction can be regarded as additional criterion for overcoming of resistance next to high ELISA values.

Since only the effect of inoculum concentration of BNYVV and *P. betae* were considered for closer investigations in this study we excluded the discussion about increased pathogenicity due to presence and genetic variability of a fifth RNA as occurring in the P-type. Additional, we

abstracted away from considerations on higher pathogenicity of BNYVV-A-types coming along with high variability in the amino acid tetrad position 67-70 on the pathogenicity factor P25 encoded by RNA3. Especially the presumed connection of valine on position 67 of P25 and the ability for these BNYVV-A-types to overcome resistance could not be supported in recently published articles (29), what lead us to disregard further investigations in our studies.

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LIST OF PUBLICATIONS

Papers

PFERDMENGES, F, KORF, H. AND VARRELMANN, M. (2007). Identification of Rhizomania infected soil in Europe able to overcome *Rz1* resistance in sugar beet and comparison to other resistance breaking soils from different geographic origins. *European Journal of Plant Pathology*; manuscript submitted.

PFERDMENGES, F. AND VARRELMANN, M. (2007). Resistance breaks in soils from Europe and the USA are independent from BNYVV (*Beet necrotic yellow vein virus*) and *Polymyxa betae* inoculum densities. *Plant Disease*; manuscript submitted.

PFERDMENGES, F. AND M. VARRELMANN (2006) Wird Rizomania wieder ein Problem im Zuckerrübenanbau? Beobachtungen von auftretender Resistenzüberwindung im Ausland. *Zuckerrübe 55*, 266-268

KÖNIG R., G. BÜTTNER, F. PFERDMENGES, W. HERRENSCHWAND, G. DEML AND M. VARRELMANN (2005) Distribution of various types of *Beet necrotic yellow vein virus* in Europe and abroad. 6. Symposium of the International Working Group on Plant Viruses with Fungal Vectors, 05.-09. 09. 2005 – Bologna

Presentations

PFERDMENGES, F. Appearance, spread and pathogenicity of different forms of *Beet necrotic yellow vein virus* (BNYVV) Institut für Phytopathologie, 02.03.2005 – Universität Göttingen

PFERDMENGES, F. Vorkommen, Ausbreitung und Pathogenität verschiedener Formen des Rizomaniavirus (*Beet necrotic yellow vein virus* (BNYVV). GFP-Jahrestagung, 03.11.2005 – Bonn

PFERDMENGES, F. AND M. VARRELMANN Appearance, spread and pathogenicity of different forms of *Beet necrotic yellow vein virus* (BNYVV) BNYVV variability meeting, 08.12.2005 - Paris

PFERDMENGES, F. Resistenzüberwindung von Zuckerrübenhybriden (*Rz*1 x *Rz*2) durch *Beet necrotic yellow vein virus* (BNYVV) P-Typ nach mechanischer Inokulation. DPG-Arbeitskreis „Pflanzenvirologie“, 29. -31. März 2006 - Freudenstadt-Lauterbad

PFERDMENGES, F. AND M. VARRELMANN Standardisation of virus inoculum to evaluate pathogenicity of different BNYVV strains. IIRB Study group on "Pests and Diseases”, 24.-26.05.2006 – Sevilla

PFERDMENGES, F. Hypotheses for severe Rhizomania symptoms on sugar beet and breaking of resistance especially in Spain and the USA. BNYVV variability meeting, 01.09.2006 - Louvain-La-Neuve

PFERDMENGES, F. Vorkommen, Ausbreitung und Pathogenität verschiedener Formen des Rizomaniavirus *(Beet necrotic yellow vein virus*, BNYVV). GFP-Jahrestagung, 08.11.2006 – Bonn

PFERDMENGES, F. AND M. VARRELMANN. Nachweis der variablen Pathogenität von Rizomania (*Beet necrotic yellow vein virus*, BNYVV) und dem Vektor *Polymyxa betae* gegenüber verschiedenen Zuckerrübengenotypen, DPG - Arbeitskreis „Pflanzenvirologie“ 30.03.2007 – Quedlinburg

PFERDMENGES, F. AND M.. VARRELMANN. Characterisation of soils from different geographic origin containing *Beet necrotic yellow vein virus* (BNYVV) which overcomes *Rz*1 resistance in sugar beet. 34th General Meeting of the ASSBT, 28.02. – 03.03. 2007 - Salt Lake City

PFERDMENGES, F. AND M.. VARRELMANN Characterisation of soils from different geographic origin containing *Beet necrotic yellow vein virus* (BNYVV) which overcomes *Rz*1 resistance in sugar beet. BNYVV variability meeting, 26.04.2007 - Paris

PFERDMENGES, F. Characterisation of soils from different geographic origin containing *Beet necrotic yellow vein virus* (BNYVV) which overcomes resistance in sugar beet
Institut für Phytopathologie, 26.06.2007 – Universität Göttingen

Poster

PFERDMENGES F., R. KOENIG UND M. VARRELMANN. *Beet necrotic yellow vein virus* (BNYVV) – P-Types overcomes resistance in *Rz*1+*Rz*2 sugar beet hybrids after mechanical inoculation. 69. IIRB-Congress, 15.-16.02.2006 - Brüssel

PFERDMENGES F., R. KOENIG UND M. VARRELMANN *Beet necrotic yellow vein virus* (BNYVV) – P-Types overcomes resistance in *Rz*1+*Rz*2 sugar beet hybrids after mechanical inoculation. Advances in Plant Virology Warwick University, 5-7 April 2006 - Warwick
- 3rd Poster price -

PFERDMENGES, F. UND M. VARRELMANN Bestimmung der Inokulumdichte von Isolaten des *Beet necrotic yellow vein virus* und Nachweis der variablen Pathogenität gegenüber verschiedenen Zuckerrübengenotypen - Resistenztest in natürlich infiziertem Boden versus *Polymyxa betae* Zoosporeninfektion. 55. Deutsche Pflanzenschutztagung., 25. - 28. September 2006 - Göttingen

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Eidesstattliche Erklärung

Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Friederike Pferdmenges

Göttingen, im September 2007