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RNA silencing mechanisms are responsible for outstanding resistance of some wild beets against rhizomania. A preliminary evidence-based hypothesis

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1 ABSTRACT

Wild relatives of sugar beet constitute a reservoir of many valuable traits that may support breeding of the crop. One broadly known example of such implementation may be *Rz2*, a gene encoding rhizomania resistance that originates from wild beet *B. vulgaris* ssp. *maritima* and was successfully employed in breeding programmes of sugar beet. The aim of this study was to reflect biodiversity of some genebank selected accessions of *B. vulgaris* ssp. *maritima* and *B. vulgaris* ssp. *vulgaris* in the context of rhizomania resistance and to evaluate the possible basis of their resistance. To reach these goals we used a broad range of molecular techniques including RT-PCR and real-time RT-PCR for the presence and quantification of *Polymyxa betae* and BNYVV, PCR with literature-based rhizomania resistance segregating sequences, RT-PCR and HRM screening for the identification of putative components of the virus-vector-host interaction, DNA laddering method for detection of DNA fragmentation, as well as microscopic and morphological observations. Our report documents some unique resistance source related features, but also discusses possible mechanisms of resistance development with reference to the results obtained.

2 INTRODUCTION

Rhizomania is a disease of beet caused by BNYVV (*Beet necrotic yellow vein virus*). The disease had spread worldwide long before the true pathogenic agent was recognized, contributing as a result to significant quantitative and qualitative losses of the sugar beet yield (Chiba *et al.*, 2011; Pavli *et al.*, 2011). The only effective method protecting against symptom development in infested areas is thought to be the cultivation of tolerant sugar beet plants. Because breeding companies have produced partially resistant materials, it may thus seem that the problem of rhizomania has been solved and difficulties overcome. On the other hand, however, there is still a risk of a massive disease outburst in face of the appearance of new and severe resistance-breaking viral pathotypes. Therefore, a continuous production of competitive commercial cultivars requires identification and combination of different resistance sources into one cultivar (De Biaggi *et al.*, 2010). The resistance sources described thus far, which are the most broadly exploited in the breeding programmes are Rz1 and Rz2 and they may probably have originated from some wild beet ancestors (Biancardi *et al.*, 2002). However, other

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sources, designated as Rz3, Rz4 and Rz5 were also found in *Beta vulgaris* ssp. *maritima* (Gidner *et al.*, 2005; Grimmer *et al.*, 2007; Grimmer, *et al.* 2008), although their genetic independence has not been fully verified yet. Evaluation of different accessions of *Beta vulgaris* ssp. *vulgaris* and *Beta vulgaris* ssp. *maritima* may thus constitute a prerequisite of successful selection and determination of new resistance sources (Frese, 2003; Luterbacher *et al.*, 2005; Panella and Lewellen, 2007). The aim of this work was to reflect biodiversity of some genebank-selected accessions of *Beta vulgaris* ssp. *maritima* and *Beta vulgaris* ssp. *vulgaris* in the context of rhizomania resistance. This is based on their predetermined phenotype of resistance/susceptibility, according to the genebank information. The second purpose was also to detect some elements that feature different structure or are differently regulated during a host-pathogen interaction between different sources of resistance/susceptibility as well as to establish whether these preliminary results could give us some insight into a possible basis of the resistance in the accessions under study.

3 MATERIALS AND METHODS

3.1 Plant material: The following accessions, based on the database and/or literature information, were expected to be highly resistant/tolerant, were included in the study: WB151, Denmark (resistance score: 1; selected from GRIN - the Germplasm Resources Information Network at the Agricultural Research Service of the United States Department of Agriculture), WB191, Denmark (resistance score: 1; GRIN), 45512, Greece (resistance score: 1; IDBB - the International Database for Beta) and WB42, a putative *Rz2* carrying resistance source, the USA (resistance score: unknown; GRIN). Apart from these highly resistant/tolerant accessions, a group of susceptible plants was also selected from GRIN, including WB242, France (resistance score: 4; GRIN), WB531, Italy (resistance score: 7; GRIN) and WB580, Italy (resistance score: 8; GRIN). Additionally, the accession 2/2 belonging to the PBAI- NRI (Plant Breeding and Acclimatization Institute - National Research Institute) collection had previously unspecified disease descriptors (resistance score: unknown; PBAI-NRI). The ploidy level of the obtained materials was confirmed cytometrically. Reference sugar beet cultivars 'Japola' (susceptible) and 'Lessing' (resistant) were included for calibration in quantitative comparisons.

3.2 *In vitro* plant culture: To provide enough amount of homogenous plant material for further analyses, individuals obtained from single seeds for each accession were propagated *in vitro*. Apical shoots were briefly sterilized and cultured in MS (Murashige and Skoog, 1962) medium containing 1 mg/l BAP and 0.1 mg/l NAA. Incubation of the explants in the growth room (25°C, 16-h photoperiod, light intensity: 40 mol/m²s) resulted in the formation of fully developed sprouts. Then they were transferred into MS medium with 0.3 mg/l BAP and 0.1 mg/l NAA. After the propagation phase, regenerated explants were cultured in the rooting medium containing 3 mg/l IBA. During the phase of *in vitro* propagation the observations regarding morphological features of the root system were performed.

3.3 Infestation of the plant material: The rooted individuals were acclimatized in the greenhouse and subsequently kept in the growth room (22°C day, 10h; 17°C night, 14h) in ceramic pots filled with a mixture of soil containing BNYVV pathotype A originating from a known BNYVV-infested field in Śmiłowo, sand and peat (2:1:6). The presence of *P. betae* and BNYVV was confirmed in an initial experiment microscopically and by RT-PCR, respectively. After an 8-week infestation period, root samples were collected for further experimental procedures.

3.4 Microscopic observation-based evaluation of *Polymyxa betae*: The roots of three individuals per accession were checked for the presence of *Polymyxa betae* by direct examination under a light microscope (Jenamed 2, Carl Zeiss). Small pieces of rootlets were cut out from the plant, washed and mounted in a mixture of ethanol, glycerine and distilled water (1:1:1). The results were expressed as the percentage and mean number of resting spores in 30 pieces of the rootlets (about 1 cm long) per each plant.

3.5 RNA isolation and reverse-transcription: RNA isolation protocol was performed using GeneMATRIX Universal RNA Purification Kit (EURx Ltd.), according to the manufacturer's instruction, supplemented with an extended version

of DNase I treatment step (DNase I, Fermentas). Purity and concentration of RNA samples were examined at NanoDrop 2000c Spectrophotometer (Thermo Scientific). 300 ng of RNA was used for first strand cDNA synthesis in a 20 µl reaction volume. Reaction mixture contained also: oilgo(dT)$_{18}$ primer (Genomed), dNTP Mix 10 mM each (Thermo Scientific Fermentas), 5x RT Buffer (Fermentas), RiboLock™ RNase Inhibitor (Fermentas) and RevertAid™ Reverse Transcriptase (Fermentas) in the final concentrations as described by the reverse transcriptase manufacturer. The samples were incubated for 60 min at 42°C, which was followed by a termination step (85°C, 5 min).

3.6 Reverse-transcription PCR: Before reaction, the concentration of cDNA was normalized in each sample to the basal level of about 600 ng/µl. Reverse-transcription PCR was performed using 4 µl of a template and the following substrates: $MgCl_2$ (Thermo Scientific Fermentas; final concentration: 3.125 mM), dNTP (Thermo Scientific Fermentas; 0.25 mM), specific primers (Genomed; 1.25 µM), DreamTaq Polymerase (Thermo Scientific Fermentas; 0.05 u/µl), 10x *Taq* Buffer with $(NH_4)_2SO_4$ (Thermo Scientific Fermentas; 1x) and DEPC-treated water (Genoplast) up to 20 µl. Basic reaction programme was: initial denaturation (94°C, 2 min), 35 cycles of denaturation (94°C, 1 min), annealing (50-63°C - depending on the set of primers used, 1 min), elongation (72°C, 1 min 30 s - 3 min, depending on the size of expected product) and final elongation (72°C, 3 min).

3.7 Real-time RT-PCR for the quantification of BNYVV and *Polymyxa betae* : Experiments were performed on PikoReal™ Real-Time PCR System (Thermo Scientific), using FastStart Essential DNA Green Master (Roche Applied Science). In the first step, standard curves were delineated, based on serial template dilutions, to establish -reaction efficiency for each gene under investigation. Then, the Relative Quantification analysis allowed us to calculate target/reference gene ratios for each of the samples. Glutamine synthetase and actin were employed as the reference genes. Their primer sequences as well as the primer sequences for the target genes either were found in the databases or designed using Primer BLAST (NCBI). **HRM analysis:** Experiments were performed on PikoReal™ Real-Time PCR System (Thermo Scientific), using *Precision*™ HRM Mastermix (PrimerDesign Ltd.). Primer sequences were designed using Primer BLAST (NCBI).

3.8 Genomic DNA isolation, PCR and electrophoresis: DNA isolation from leaves was carried out as described previously (Davis *et al.*, 1986). The same procedure was applied for detection of DNA fragmentation (the DNA laddering method) in the root tissue. The concentration and integrity of obtained DNA samples were evaluated spectrophotometrically and electrophoretically (2.5 µg of DNA per well). Some rhizomania-segregating sequences were selected from the literature and their presence in the analysed materials was examined. PCR mixtures were as follows: 1 ng/µl DNA, 2.5 mM $MgCl_2$ (Thermo Scientific Fermentas), 0.2 mM dNTP (Thermo Scientific Fermentas), 1 µM specific primers (Genomed), 0.028 u/µl DreamTaq Polymerase (Thermo Scientific Fermentas), 1x *Taq* Buffer with $(NH_4)_2SO_4$ (Thermo Scientific Fermentas) and PCR grade water up to 20 µl. Different reaction programmes were applied, depending on the conditions previously described for a given primer sequence. Amplification products were resolved on 1.5% agarose (Prona Agarose, Basica Le) stained with ethidium bromide (Promega; 0.5 µg/ml), at 5 V/cm of gel. The size of the products was estimated using Gel Doc™ 2000 Gel Documentation System (Bio-Rad Laboratories Srl, Milan, Italy) equipped with Quantity One, version 4.0.3 software, by comparison with the standard, i.e. GeneRuler™ 100 bp Plus DNA Ladder (Thermo Scientific Fermentas). All the sequences of primers included in the study are available upon request.

3.9 Observations of survivors: The percentages of surviving/dead plants as well as their morphological features were evaluated following a 6-month growth period in the infested soil.

4 RESULTS

There were visible differences observed in the morphology of leaves, colour of the stem and leaves and, especially, colour of the root system among the studied plants growing in *in vitro* cultures (Fig. 1a-h').

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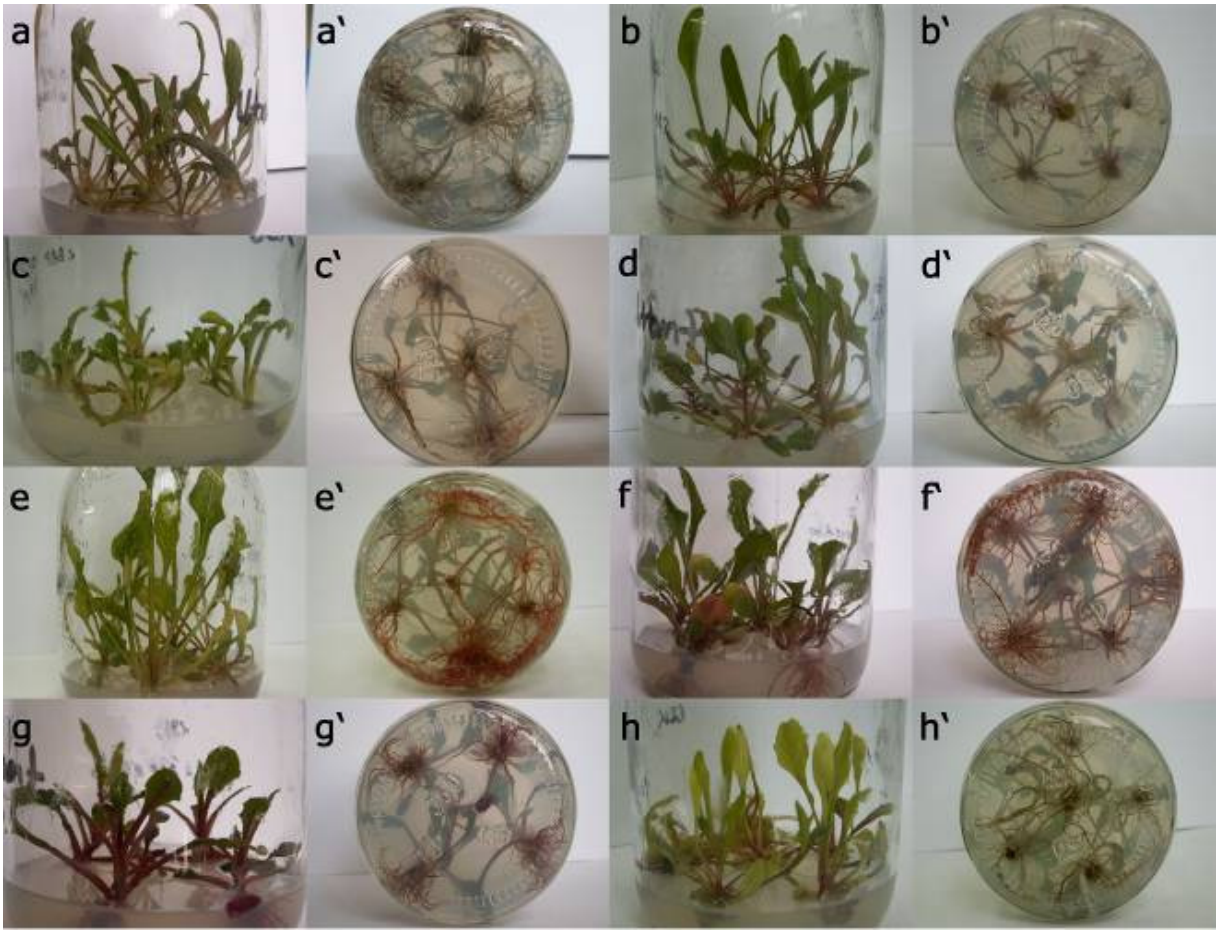

Figure 1: Visible morphological differences between the analysed accessions growing in *in vitro* cultures. A,A' - WB580, B,B' - 45512 IDBB, C,C' - 2/2 PBAI-NRI, D,D' - WB242, E,E' - WB191, F,F' - WB151, G,G' - WB42, H,H' - WB531

Three out of eight analysed accessions, i.e. WB42, WB151 and WB191 had red roots (Fig. 1e',f',g'), whereas others presented white, brown or grey and only rarely reddish roots. Interestingly, however, the colouration of the root system disappeared during the growth of plants in infested soil, being comparable for the resistant and susceptible accessions (Fig. 2).

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Figure 2: Morphological features of selected accessions under study after a period of growth in infested soil - the root system. A - WB191, B - WB151, C - WB42, D - WB242

At further stages of development, it was also possible to recognize two basic groups of plants, in reference to their foliage. Thin and longitudinal blades with long petioles were typical for resistant materials (Fig. 3a-d), whereas more thick and fleshy leaves appeared in susceptible ones (according to the genebank score) (Fig. 3e-h).

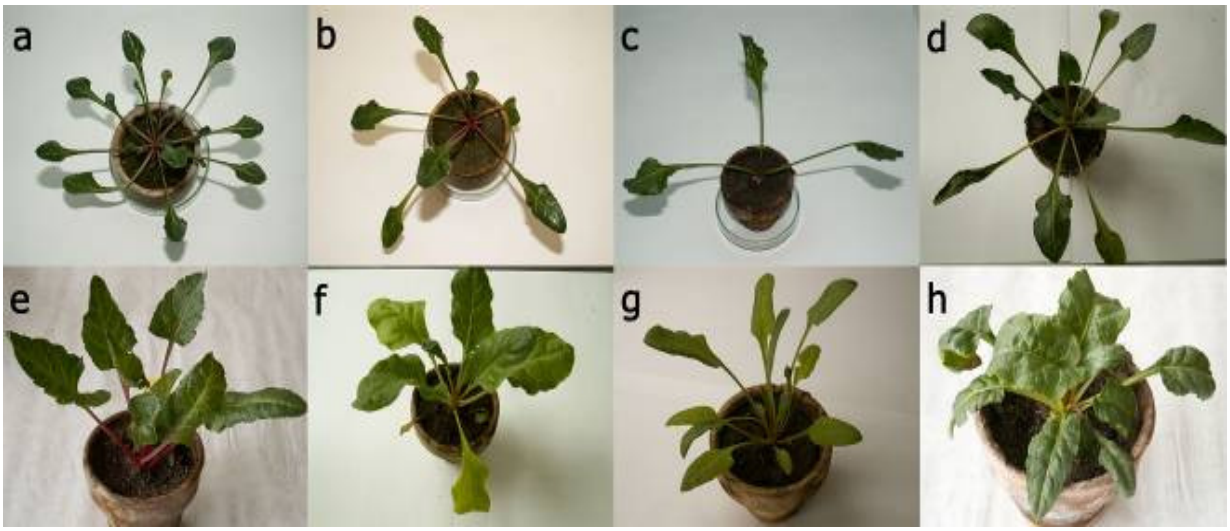

Figure 3: Morphological features of the accessions under study after a period of growth in infested soil - leaf and leaf rosette traits. A - WB151, B - WB42, C - WB191, D - 45512 IDBB, E - WB242, F - WB531, G - WB580, H - 2/2 PBAI-NRI

RT-PCR for the presence of BNYVV was performed using specific primers that amplify a 500-bp fragment of the read-through region of the coat protein gene, located in RNA-2 component of the genome. This experiment allowed us to differentiate materials under the study into two groups, manifesting either high or low quantity of the virus in their roots (Fig. 4a). Apart from RNA-2, also other components of the viral genome typical for BNYVV pathotype A, i.e. RNA-1, RNA-3 and RNA-4 were examined, yielding similar results (Fig. 4b-d). Strikingly, however, RNA-3 and RNA-4 fragments were undetectable/on the verge of the detection limit in most of the accessions that showed the lowest amount of RNA-1 and RNA-2 components as well, i.e. in WB42, WB151 and WB191, similarly to RNA-1 in WB151 (Fig. 4b-d). The presence of these barely detectable fragments was nevertheless confirmed in about one third of the samples for RNA-3 and RNA-4 in WB42 and WB191, as well as for RNA-1 in WB151. RNA-3 and RNA-4 fragments have never been found in WB151 and RNA-1 was always present in WB42 and WB191 (Fig. 4).

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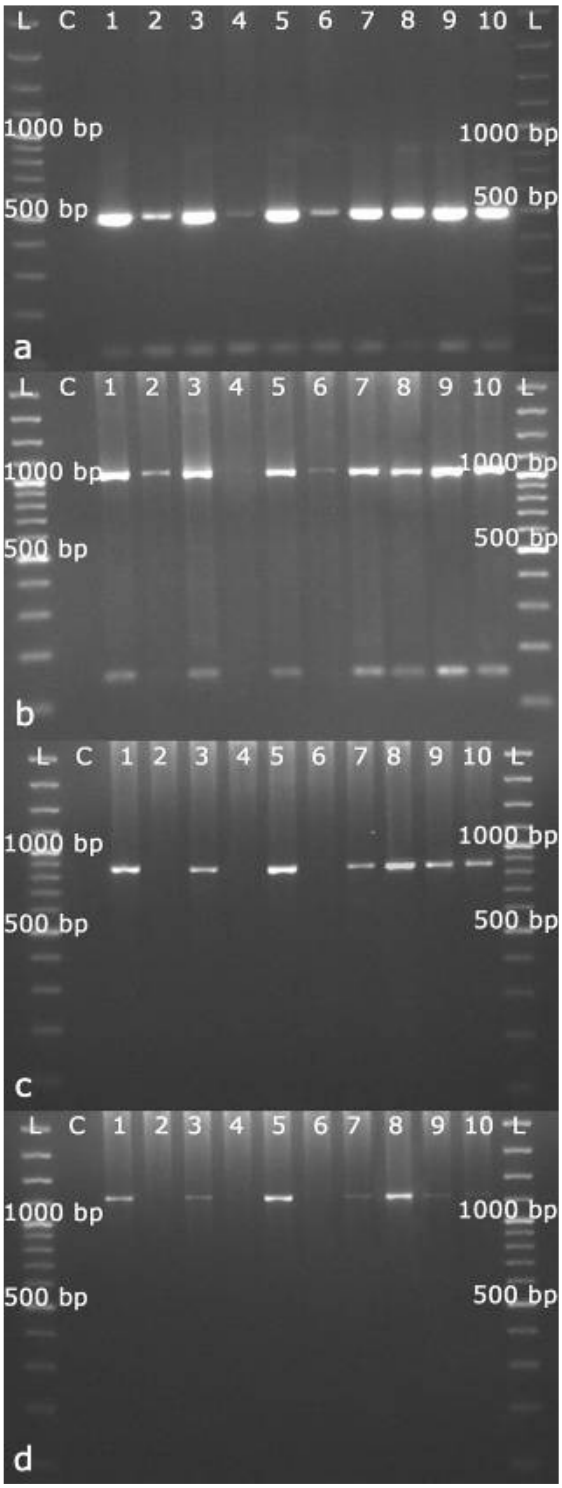

Figure 4: RT-PCR for the presence of specific components of BNYVV genome in roots of the materials under study after growth in infested soil. A - RNA-2, B - RNA-1, C - RNA-3, D - RNA-4. L - ladder, C - negative control, 1 - WB580, 2 - WB191, 3 - 45512 IDBB, 4 - WB151, 5 - 2/2 PBAI-NRI, 6 - WB42, 7 - WB242, 8 - WB531, 9 - reference sugar beet cultivar 'Japola', 10 - reference sugar beet cultivar 'Lessing'. The sizes of obtained products were 492 and 124 bp for RNA-2 and glutamine synthetase, respectively (A), 1061 bp for RNA-1 (B), 861 bp for RNA-3 (C) and 1244 bp for RNA-4 (D)

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Surprisingly, the amounts of particular RNA components seemed to be rather comparable in sugar beet cultivars, in reference with each other. On the other hand, more uniform distribution of *Polymyxa betae* among susceptible and resistant individuals was evidenced by RT-PCR, although some degree of variability in product intensities was recognizable (Fig. 5).

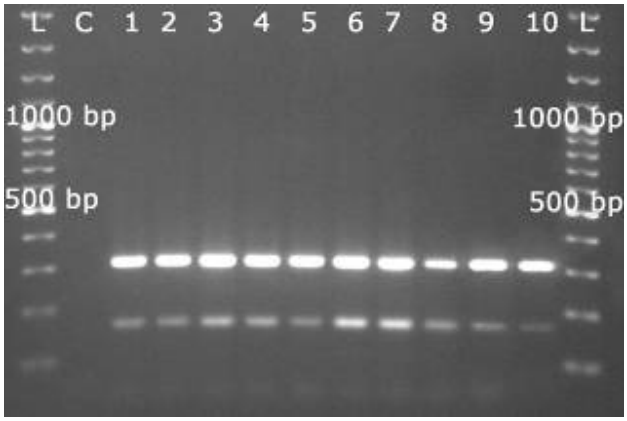

Figure 5: RT-PCR for the presence of *Polymyxa betae* in roots of the materials under study after growth in infested soil. L - ladder, C - negative control, 1 - WB580, 2 - WB191, 3 - 45512 IDBB, 4 - WB151, 5 - 2/2 PBAI-NRI, 6 - WB42, 7 -WB242, 8 - WB531, 9 - reference sugar beet cultivar 'Japola', 10 - reference sugar beet cultivar 'Lessing'. Actin was used as a reference gene. The sizes of obtained products were 174 bp and 324 bp for *P. betae* and actin, respectively

This was also in agreement with microscopic observations of the vector in the root tissue. The presence of cystosori was documented for all the accessions (Fig. 6), ranging from 80-93% for most of the plants to 63 and 40% for WB531 and WB191, respectively (Fig. 7a).

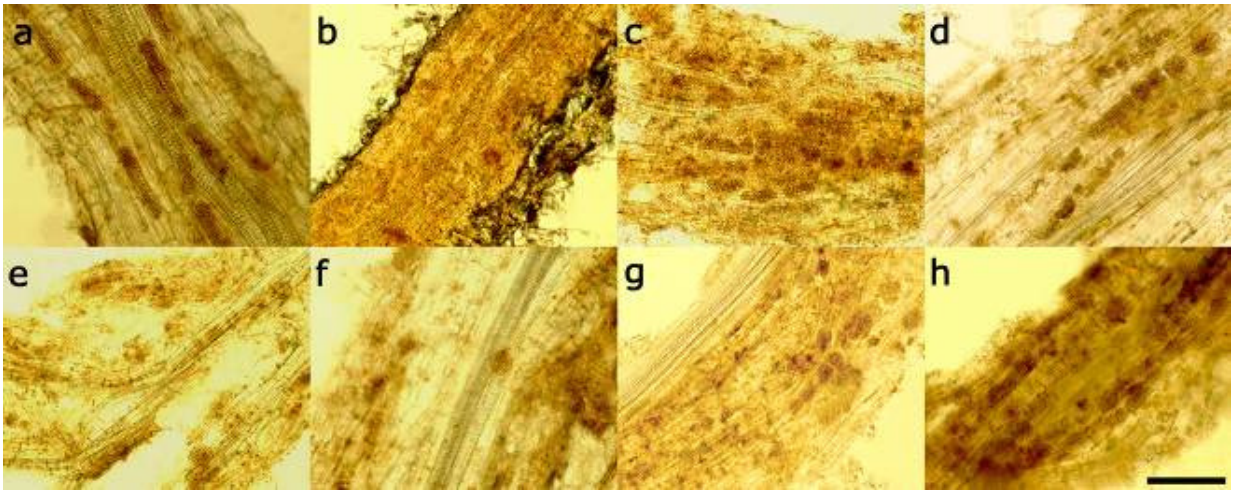

Figure 6: Microscopic observations of *Polymyxa betae* cystosori in the root tissue of analysed accessions after growth in infested soil. A - WB151, B - WB191, C - WB42, D - WB242, E - WB580, F - WB531, G - 45512 IDBB, H - 2/2 PBAI-NRI. Bar = 100 μm

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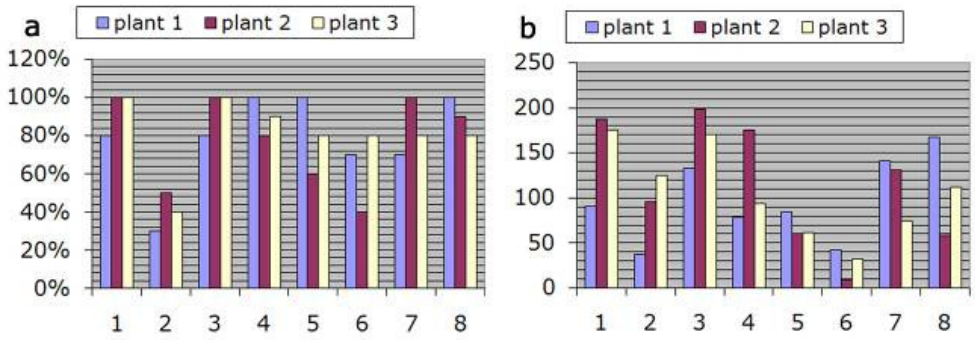

Figure 7: Microscopic observations of *Polymyxa betae* cystosori in the root tissue of analysed accessions after growth in infested soil - statistics. For each plant 30 pieces of the rootlets (about 1 cm long) were examined, three plants per accession and up to 5 independent places for each vector-positive slide were counted. A - Percentage of *Polymyxa betae*- positive slides, B - mean amount of *Polymyxa betae* resting spores for each accession under the study. 1 - WB151, 2 -WB191, 3 - WB42, 4 - WB242, 5 - WB580, 6 - WB531, 7 - 45512 IDBB, 8 - 2/2 PBAI-NRI

The lowest mean amount of resting spores featured WB531 and WB580 (Fig. 7b). In order to obtain more precise indications, real-time RT-PCR procedures were employed. These experiments revealed in turn three major groups as regards the relative BNYVV concentration, i.e. a group of high concentration (WB531 and WB580), a group of intermediate concentration (2/2 PBAI-NRI, 45512 IDBB and WB242) and a group of exceptionally low concentration represented by WB191, WB42 and WB151 (Fig. 8a). Results of the real-time RT-PCR experiment for the quantification of *Polymyxa betae* showed also some degree of variation in the relative content of the vector among the studied accessions. The highest values were obtained for WB242 and WB531, the lowest for WB191, 45512 IDBB and 2/2 PBAI-NRI, whereas intermediate concentrations were found in WB42, WB580 and WB151 (Fig. 8b).

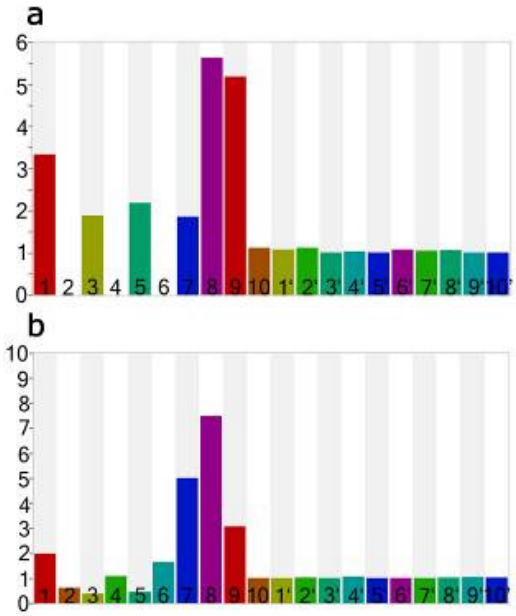

Figure 8: Real-time RT-PCR analysis for the presence of rhizomania-mediating agents. A - Relative quantification of BNYVV (1-10) with reference to glutamine synthetase (1'-10'), B - relative quantification of *Polymyxa betae* (1-10) with reference to actin (1'-10'). 1,1' - WB580, 2,2' - WB191, 3,3' - 45512 IDBB, 4,4' - WB151, 5,5' - 2/2 PBAI-NRI, 6,6' -WB42, 7,7' - WB242, 8,8' - WB531, 9,9' - reference sugar beet cultivar 'Japola', 10,10' - reference sugar beet cultivar 'Lessing'

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In our experiment, the percentage of survivors turned out to be less consistent with the genebank classification than the real-time PCR based quantification (Table 1). Nevertheless, both procedures suggest most probably a misleading description as regards the resistance score in case of 45512 IDBB, at least for the clone studied here.

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Table 1 BNYVV quantity in the analysed accessions in comparison with genebank data and plant survival

Genebank accession - descriptors	Taxonomy	Resistance according to genebank	Plant survival (% of dead)	Mean relative *BNYVV* quantity
PI 546397, WB151, Denmark (GRIN[1])	*B. vulgaris* ssp. *maritima*	1	0	0.0003
PI 546385, WB42, USA (GRIN[1])	*B. vulgaris* ssp. *maritima*	unknown	0	0.001
PI 546412, WB191, Denmark (GRIN[1])	*B. vulgaris* ssp. *maritima*	1	20	0.002
PI 546413, WB242, France (GRIN[1])	*B. vulgaris* ssp. *maritima*	4	0	1.3145
45512, Greece (IDBB[2])	*B. vulgaris* ssp. *maritima*	1	100	1.8987
2/2 PBA-NRI[3]	*B. vulgaris* ssp. *maritima*	unknown	60	2.0043
PI 504250, WB580, Italy (GRIN[1])	*B. vulgaris* ssp. *maritima*	8	40	2.9697
PI 504174, WB531, Italy (GRIN[1])	*B. vulgaris* ssp. *vulgaris*	7	70	5.2025

[1] - the accession selected from the Germplasm Resources Information Network, Agricultural Research Service, the United States Department of Agriculture; [2] - the accession selected from the International Database for Beta; [3] - the accession belonging to the collection of the Plant Breeding and Acclimatization Institute - National Research Institute

In addition, some polymorphisms were identified between highly resistant/tolerant and susceptible accessions, using literature-selected resistance segregating sequences in the course of PCRs. Some of the products were obtained only for the resistant accessions (Fig. 9a-e), e.g. a PCR product for the resistance gene analogue Rz-C (Fig. 9a) that is believed to participate in a hypersensitive response. These fragments may thus be in coupling phase with resistance, whereas other bands were found only in susceptible plants, suggesting their linkage in repulsion phase (Fig. 9e,f).We were not able to document here the presence of transcriptional products for Rz-C (Fig. 10).

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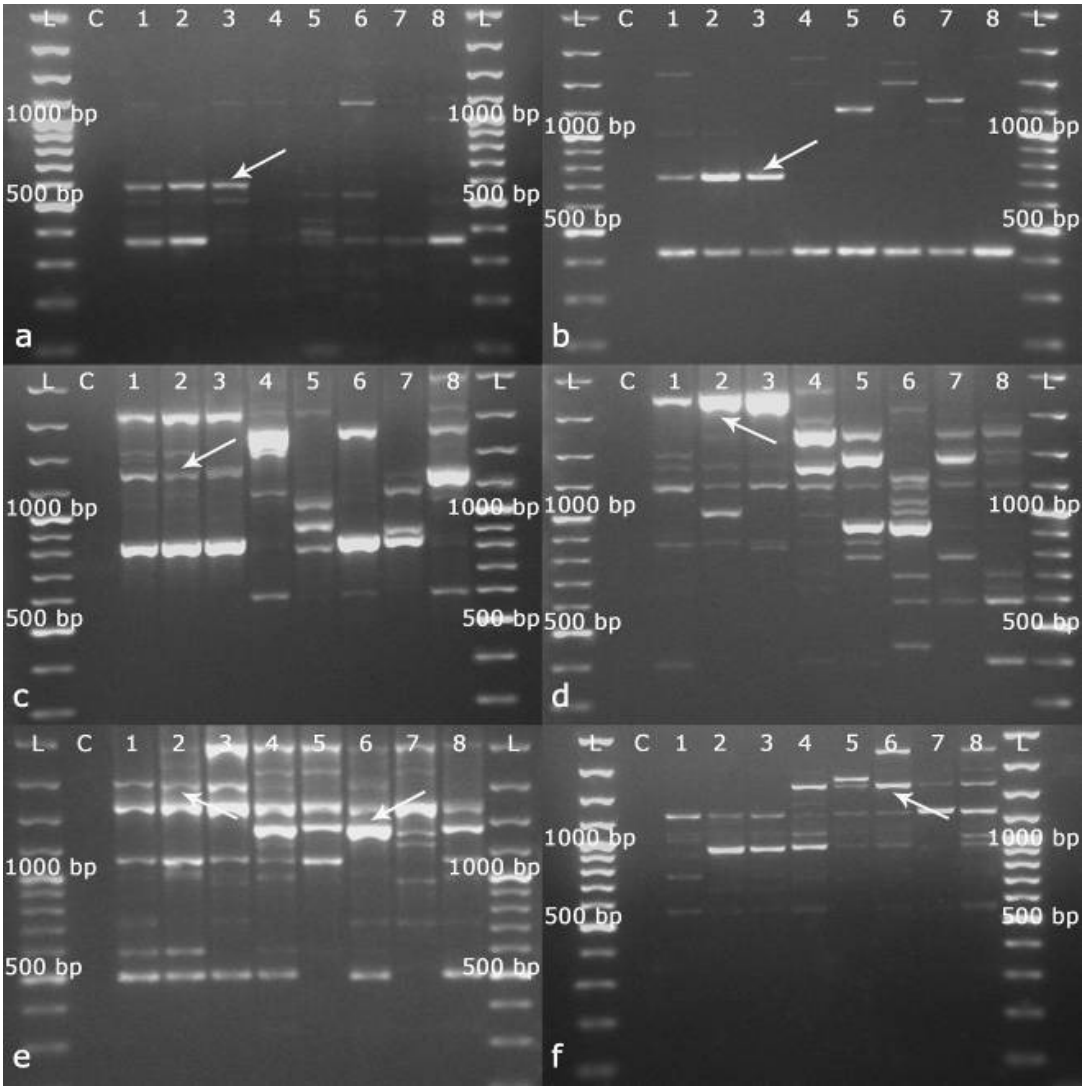

Figure 9: PCR analysis for the presence of selected resistance-segregating sequences in DNA of the accessions under study. A - primers SS-31, B - primer SS-18, C - primer SS-26, D - primer SS-27, E - primer SS-23, F - primer SS-30. L - ladder, C - negative control, 1 - WB151, 2 - WB191, 3 - WB42, 4 - WB242, 5 - WB580, 6 - WB531, 7 - 45512 IDBB, 8 - 2/2 PBAI-NRI

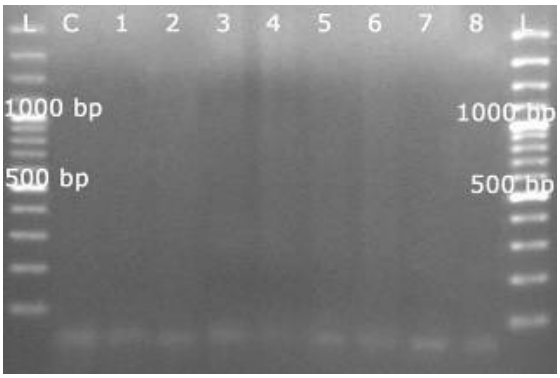

Figure 10: RT-PCR analysis for the presence of Rz-C transcriptional product in roots of the materials under study after growth in infested soil. L - ladder, C - negative control, 1 - WB151, 2 - WB191, 3 - WB42, 4 - WB242, 5 - WB580, 6 -WB531, 7 - 45512 IDBB, 8 - 2/2 PBAI-NRI

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Interestingly, in the course of our high resolution melting analysis we confirmed the presence of different variants in the DNA sequence of a putative FBK (kelch repeat containing F-box family proteins) between susceptible and highly resistant accessions. For one of the sequences tested the difference was visible mainly for 45512 IDBB (Fig. 11a,b), but the other fragment showed an interesting HRM profile, being clearly distinct for the group of highly resistant materials as compared to the susceptible group (Fig. 12a,b). Surprisingly however, it seems that the sequence difference between highly resistant WB151 and the susceptible plants was not so evident as for WB42 and WB191, which may reflect unique features of particular resistance sources (Fig. 12a,b).

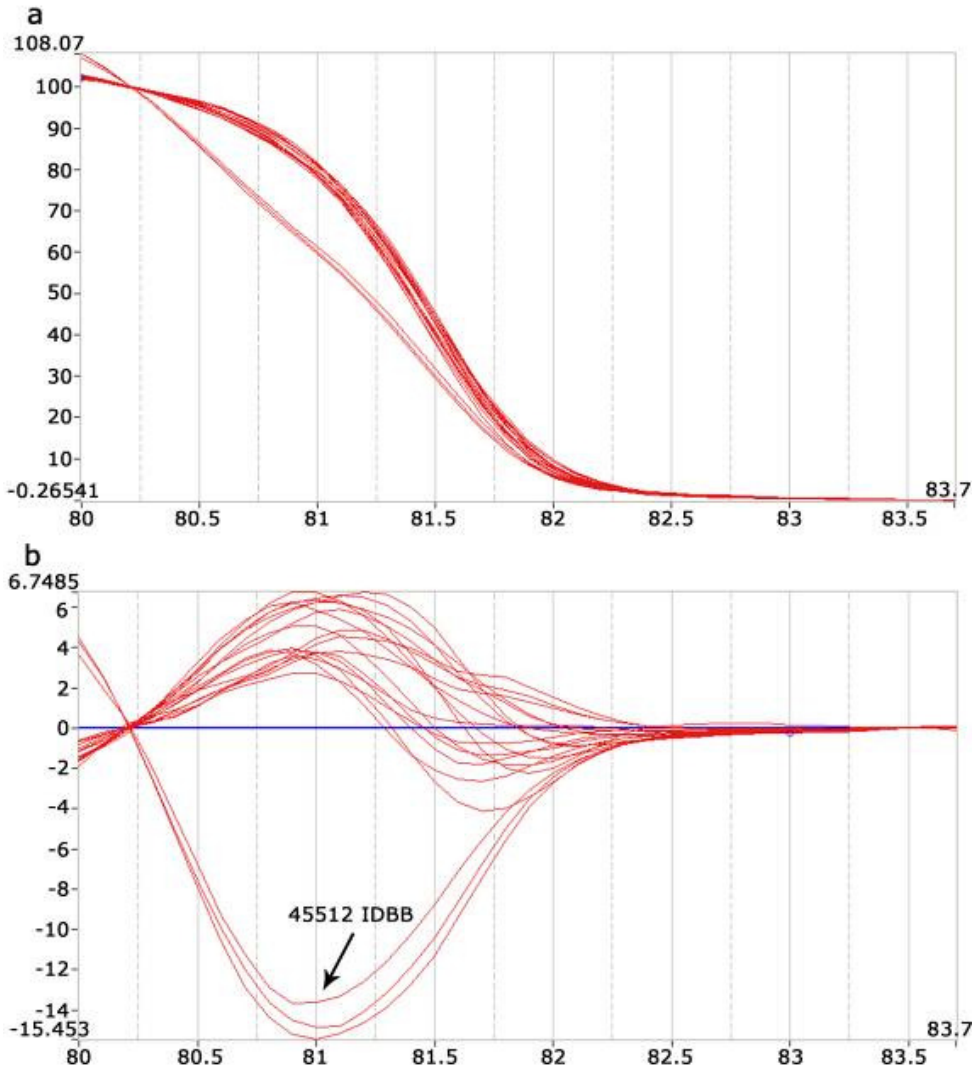

Figure 11: HRM analysis of a candidate sequence for the host pathogen interaction of the accessions under study, primers SS-37. A - HRM graph (relative fluorescence units versus temperature), B - difference graph (difference versus temperature). Considerable difference was found only for 45512 IDBB, as indicated by the arrow

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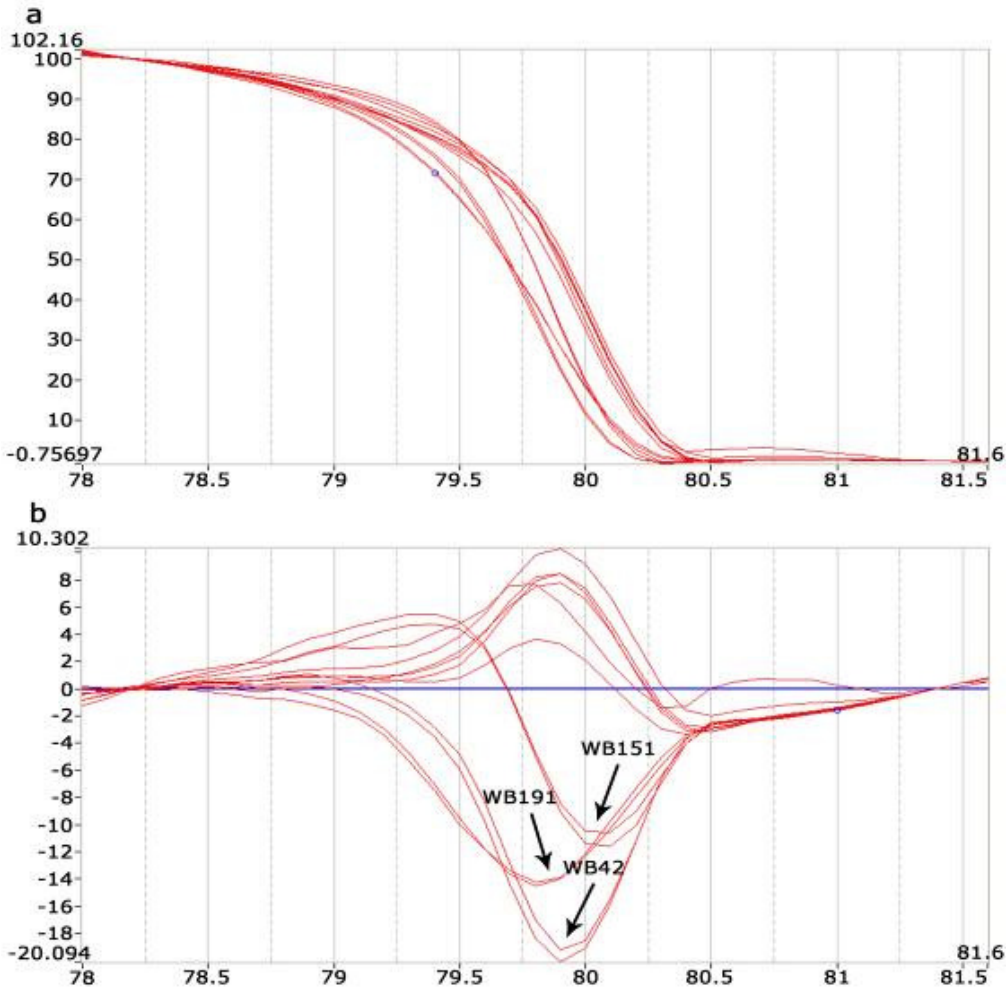

Figure 12: HRM analysis of a candidate sequence for the host pathogen interaction of the accessions under study, primers SS-38. A - HRM graph (relative fluorescence units versus temperature), B - difference graph (difference versus temperature). Considerable differences were found for highly resistant accessions, as indicated by arrows

Agarose gel electrophoresis that was performed on DNA samples isolated form roots did not reveal typical ladder-like pattern (Fig. 13).

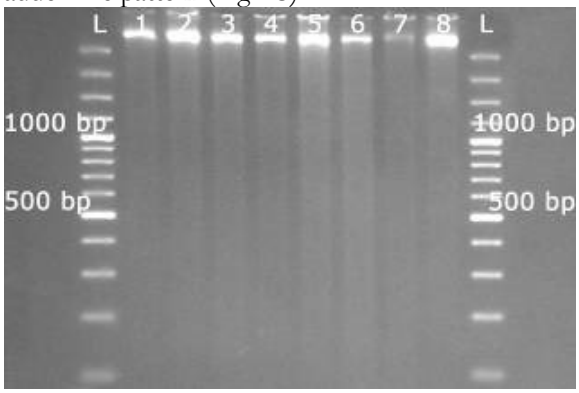

Figure 13: Agarose gel electrophoresis - detection of DNA fragmentation patterns. 1 - 2/2 PBAI-NRI, 2 -WB151, 3 - WB191, 4 - WB42, 5 - WB242, 6 - WB580, 7 - WB531, 8 - 45512 IDBB

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Instead, similar patterns of a faint smear were evident for each of the accessions under the study, which does not confirm oligonucleosomal DNA fragmentation at this stage of the experiment.

DISCUSSION

Different sources of resistance to rhizomania disease have been identified among wild beets, especially *Beta vulgaris* ssp. *maritima*, including accessions that manifest BNYVV- and/or *Polymyxa betae*-directed protective mechanisms (Scholten and Lange, 2000; Asher *et al*., 2009). There are also studies aiming at gene mapping in the course of which some closely-linked markers as well as a possible mode of heredity are being described in mapping populations. Based on the results of the above-mentioned experiments, it has been established that the hypothesis of a single gene dominance does not fully explain the variability of phenotypes, at least in some populations (Pelsy and Merdinoglu, 1996; Giorio *et al*., 1997; Scholten *et al*., 1997; Wisler *et al*., 1999; Gidner -*et al*., 2005; Lein *et al*., 2006). Nevertheless, the potential resistance genes found in particular sources were distinguished and designated as follows: *Rz1*, *Rz2*, *Rz3*, *Rz4* and *Rz5* (Pelsy and Merdinoglu, 1996; Scholten *et al*., 1999; Gidner *et al*., 2005; Grimmer *et al*., 2007; Grimmer *et al*., 2008). However, , it seems unclear whether they represent independent loci or constitute, at least some of them, allelic variants of one another. The distinction was evident only in case of *Rz1* and *Rz2* (Scholten *et al*., 1999; Amiri *et al*., 2003), whereas for other sequences it is presumed that they most probably cover the same/overlapping positions in the genome (Grimmer *et al*., 2007; Grimmer *et al*., 2008; Pavli *et al*., 2011).

At the same time, however, the information concerning not only possible source-associated specific, but also general mechanisms that underlie the resistance development in its natural reservoirs is currently limited, although it is known that the field performance of some accessions is much better than others under severe infestation conditions and/or in the presence of extremely vicious pathotypes of the virus (Panella and Lewellen, 2007). It seems thus that elucidation of physiological functions of the genes and more precise characterization of the mechanisms crucial for the resistance response in different sources are required for successful combination of many resistance mediators in one cultivar. In order to reach this purpose, some studies have been devoted to identification of different targets of resistance. Primary suggestions related to *Polymyxa betae*-directed mechanisms are based on analyses performed in the *Procumbentes* section (Paul *et al*., 1992; Barr *et al*., 1995) in which the protective mechanisms acting at the beginning of the infectious cycle were ascribed to the hypersensitive response (Barr *et al*., 1995). Analogical barriers may also function in some individuals belonging to the *Corollinae* section as well as in some accessions of *B. vulgaris* ssp. *maritima* (Paul *et al*., 1993; Kingsnorth *et al*., 2003). Bearing in mind that some *Rz* genes may be allelic or at least closely linked, this direction of studies seems fully justified, all the more so because an additive effect of this type of resistance in combination with *Rz1* has actually been proven (Grimmer *et al*., 2008; Asher *et al*., 2009).

Here is the report of the results of this study regarding different quantities not only of the virus, but also of the vector in selected resistance sources, including WB42, WB151 and WB191 in comparison with a group of susceptible plants. This, in turn, allowed us to reflect on the biodiversity existing in wild beet accessions in reference to standard cultivars, but also to make some observations regarding the differential contribution of each of the mediating pathogens, as well as individual viral RNA components to the final phenotype. As regards this study's relative real-time PCR experiment for the quantification of *Polymyxa betae*, it seems that apart from BNYVV resistance/tolerance, some *Polymyxa betae*-directed protective mechanisms may also be involved in the development of resistance, at least in some of the analysed accessions, especially in those representing intermediate virus concentrations, like 45512 IDBB and 2/2 PBAI- NRI. However, taking into account comparable levels of *Polymyxa betae* in some highly resistant and susceptible accessions, e.g. WB42 and WB151 in comparison with WB580, it may be concluded that in the highly resistant individuals some specific virus-targeting mechanisms are particularly active. This allows us to reflect on how efficient the two basic types of resistance might be in reducing the population of the virus, with the BNYVV-targeting factors exerting the most profound effect. It is also in agreement with light microscopic experiments that documented by far not the lowest rate of *Polymyxa betae* infection in the highly resistant group of plants. Among possible causes of resistance to the virus, various potential mechanisms has been distinguished, including these related to inhibition of virus proliferation or its short-/long-distance transfer (Scholten *et al*., 1994; Tamada *et al*., 1999). On the

other hand, different components of the BNYVV genome seem to be indispensable for some specific aspects of infection. Accordingly, RNA-1 and RNA-2 encode proteins crucial for replication, encapsidation and cell-to-cell movement of the virus, whereas RNA-3, RNA-4 and RNA-5 are responsible for infection and disease manifestation in the roots and, as such, they encode proteins or non-coding elements involved in the pathogenesis, vector transmission, long distance movement as well as suppression of the plant silencing systems (Tamada *et al*., 1999; Rahim *et al*., 2007; Tamada, 2007; Peltier *et al*., 2012). Therefore, it can be assumed that different resistance mechanisms might have evolved as a host response to individual components of the pathogen genome. Bearing this in mind, this study verifiesthe presence of all of the viral components typical for pathotype A in phenotypically defined accessions of wild beet in search of some unequivocal and/or potentially divergent symptoms of resistance. The RNA silencing system is crucial for degradation of dsRNA that appears because of viral replication and/or viral sequence expression in a host cell, thus constituting part of innate immunity of plants (Maule *et al*., 2007). There are also some studies suggesting involvement of this system in the regulation of pigmentation-related mRNA (Palukaitis and MacFarlane, 2006). Interestingly, this study revealed that the plants which featured red roots during their *in vitro* culture, turned out to be the most resistant after infestation, although they lost their colouration at further stages of the study after the infestation. Whether this phenomenon is in fact somehow connected with an increased efficiency of the plant silencing system, it should nevertheless be verified in the course of extended experiments. Previously, it was documented that a kind of overlap may exist between posttranscriptional gene silencing-regulated pigment production and virus-induced silencing suppression, which is manifested phenotypically (Senda *et al*., 2004). In line with these considerations, neither a red root system nor visibly compromised suppressor molecules were observed for reference cultivars tested in our study, thus underlining a possible distinctiveness of major resistance mechanisms acting in wild beets and in cultivars. On the other hand, viruses have developed different strategies that allow them to overcome these plant protective mechanisms, affecting different aspects of their crucial steps. Many of these suppressor molecules has previously been recognized to play additional roles in the infectious cycle of a virus, like those related to multiplication, movement or symptom induction, although the detailed mechanisms of their action for many of them have to be determined yet (Palukaitis and MacFarlane, 2006; Wu *et al*., 2010). Such suppressor elements have also been recognized for BNYVV. RNA-4-encoded p31 was previously shown to be essential for efficient transmission of BNYVV via its vector, as well as for host specific symptom induction and RNA silencing suppression acting specifically in roots. The transmissibility by *P. betae* was highly dependent on the presence of full-length wild type RNA-4 in the inoculum, resulting in 100 times improved efficiency (Han *et al*., 2002; Rahim *et al*., 2007). In light of these findings it may be especially important and meaningful that here we were not able to document the presence of this component in RNA obtained from roots of infected highly resistant plants, despite its appearance in other, more susceptible accessions and despite the comparable levels of *P. betae*, which were by far not the lowest for the highly resistant accessions. Therefore, it may be hypothesized that these plants, as compared to the more susceptible genotypes, have developed particularly efficient defence mechanisms, including RNA silencing systems acting against RNA-4, which resulted in impaired virus-vector interaction. What is more, Rahim *et al*. (2007) proved also that p31 does not directly influence the level of virus accumulation in root tissue, suggesting a more complex or indirect interaction mode for this molecule, e.g. as an enhancer of RNA silencing suppression at a particular stage of infection, at which zoospores of the vector acquire the virus, thus contributing to improved transmissibility of BNYVV (Rahim *et al*., 2007). This is in agreement with our observations that the samples with undetectable amounts of RNA-4 featured concomitantly lower levels of other RNA components as well, thus supporting the concept that a kind of cause-effect relationship between individual RNA components may in fact exist *in vivo*. We believe that some differences in this field might be caused by the specificity of host-pathogen interaction, which is typical of a particular genotype, as the significant influence reflected by reduced RNA-1 and RNA-2 components was evident only for the most resistant accessions in our study. Therefore, a thorough comparison including the broad range of different resistance/susceptibility sources may serve a good exemplification of these relationships, which was the advantage of this study. Similar situation was observed by us for RNA-3. This element encodes p25, which is believed to facilitate mainly virus

multiplication and symptom induction in *Beta* species and constitutes the major pathogenicity factor in susceptible and avirulence factor in resistant plants (Koenig *et al.*, 1991; Chiba *et al.*, 2008). Some p25-interacting candidates were previously identified in a cDNA library of *Rz2* containing sugar beet, illustrating a broad range of effects that may potentially be exerted by this component. These candidate molecules belong mostly to the ubiquitination, stress and pathogen response, phytohormone and metabolic pathways, but also some targets were recognized among them that participate in symptom induction as well as translation, protein modification and gene expression (Thiel and Varrelmann, 2009). Furthermore, p25 was also recently demonstrated to participate in an interaction with a host protein presenting high degree of homology to F-box family proteins that belong to the SCF (Skp1-Cullin-F box) E3 ligase complex, thus it is believed to influence somehow the sugar beet 26S proteasome activity (Thiel *et al.*, 2012). This may suggest an impairment of the F-box protein function in the context of a hypersensitive resistance response induction, all the more so because agroinfiltration of F-box protein into *N. benthamiana* and sugar beet leaves resulted in necrosis execution. Concurrently however, coinfiltration of p25 did not lead to suppression of the symptoms. Therefore the authors hypothesized that either p25 may act on the ubiquitination system, which, in light of the above-mentioned findings, seems less probable in the aspect of HR induction, or/and that the system is used to fight viral infection. Our observations confirm especially the second scenario, as, similar to Thiel *et al.* (2012) we also were not able to demonstrate significant differences in the rate of a hypersensitive resistance response in the root system after infestation, which seem to be independent of the phenotype of resistance or susceptibility. This may rather reflect unspecific necrotic reaction related to symptom induction, which is at the same time unable to restrict the virus spread, manifesting itself by the presence of faint DNA smears instead of a typical laddering pattern that would be indicative of apoptosis. Instead, our results demonstrated clearly the differences in DNA composition of FBK between highly resistant and susceptible wild beet accessions of different genetic origins, as revealed by HRM analysis, confirming observations of Thiel *et al.* (2012) as regards the position of polymorphisms. To our best knowledge, this is the first report manifesting the presence of such sequence variants among wild beet sources that presented different resistance/susceptibility origins. At the same time, the amount of RNA-3 as well as RNA-4 components was significantly reduced in highly resistant plants, suggesting efficient silencing mechanisms acting against these suppressive elements. In this situation it may be tempting to make a suggestion that some ubiquitination/degradation functions that has not been addressed yet are rather coupled to silencing mechanisms, and that, although the interaction of p25-FBK is independent of p25 crucial amino-acid tetrad (Chiba *et al.*, 2008), the outcome of this interaction may be quite opposite, i.e. resistance response vs. resistance-breaking, e.g. depending on the structure of FBK as well, thus making probable both of the above-mentioned statements of Thiel *et al.* (2012). This suggestion may further be reinforced by another interesting hypothesis that plant viruses are able to affect the proteasome system of a host by an enhanced degradation of plant proteins, thus creating an imbalance in plant/viral components and turning it to the advantage of the virus (Banks, 2003; Thiel *et al.*, 2012). However, other results suggest that polyubiquitination is not particularly linked to silencing-suppressive activities, but it may rather reflect an unspecific response of the host defence system to the presence of viral proteins that is enhanced in the presence of some silencing suppressors (Csorba *et al.*, 2010). Thus, RNA-3-encoded p25 may influence proteolysis machinery of the host cell - in this way contributing to stability of silencing/apoptotic executors or other crucial elements of the resistance response.

However, in these preliminary observations based on DNA laddering experiment, it was neither possible to differentiate between DNA fragmentation rates nor to document changed regulation of RGA Rz-C in different resistance/susceptibility sources against BNYVV, which, along with the affected viral genome composition, may suggest a stronger involvement of the silencing than PCD pathway in the host-virus interaction. Studies that are more detailed are required in this aspect, including these concerning different stages of the infectious cycle, other oligonucleosomal fragmentation-independent modes of cell death as well as functional characterization of sugar beet FBK.

An interesting example of broadly known implications of F-box-like domain for suppression may be P0 of Polerovirus. This component was shown not only to be indispensable for suppressor function, but it is also crucial for an interaction with molecules belonging to E3 ubiquitin ligases. Thus, these observations led the

authors to suggest that an ubiquitin-mediated proteolysis machinery constitutes the effector of the virus action in the host-silencing pathway (Pazhouhandeh *et al*., 2006). What is more, further evidences have been obtained, indicating that AGO1 is a potential direct target for the proteasomal degradation, most probably mediated by an SCF complex, as its protein product, but not transcript was affected as a result of P0 ectopic expression (Bortolamiol *et al*., 2007). Nevertheless, these results were not fully consistent to date, because Baumberger and Baulcombe (2005) reported that inhibition of proteasome mediated degradation did not lead to increased AGO1 stability (Baumberger and Baulcombe, 2005). Therefore, it seems that P0 may well act as a negative regulator of the ubiquitination pathway, which, on the other hand, would be essential for the proper turnover of AGO. The suppressive activity of P0 is evident only at early stages of silencing, i.e. before RISC assembly into its mature form and it specifically relies on interference with production of siRNA/miRNA-AGO1 complexes. Additionally, this activity was demonstrated to be dependent on the presence of wild-type F-box motif, as opposed to F-box independent polyubiquitination, and, concurrently, only a minor role of proteasome in the P0-induced AGO1 degradation has been proposed (Csorba *et al*., 2010). Analogically, p25 was also suggested to interfere with substrate presentation to the SCF complex and with its subsequent degradation (Thiel *et al*., 2012). On the other hand, another important recent finding in this field has shown that RNA-3 of BNYVV includes in its structure coremin sequence, a pathogenicity factor crucial for systemic spread/long distance movement of the virus. What is more, accumulation and stabilization of non-coding RNAs following RNA-3 cleavage was dependent on the presence of wild-type coremin, implying an essential role of these RNAs in a block or saturation of the plant silencing systems (Peltier *et al*., 2012). In our opinion in this situation, it remains to be established if and how particular features of the FBK structure and/or RNA-3 coremin motif might influence efficiency of the plant RNA-silencing machinery. RNA-1 and RNA-2 BNYVV components that encode mostly housekeeping genes playing their roles in replication, encapsidation and virus movement were in our experiment detectable in highly resistant wild beet accessions, as opposed to the RNA-3 and RNA-4 suppressive elements. Although RNA-2 is known to comprise six ORFs including a 3'-terminal 14 kDa cysteine-rich protein displaying a putative posttranslational silencing suppressor activity (Zhang *et al*., 2005), in this study we amplified a different nearly 500 bp fragment of the read-through region of the coat protein gene, which is essential for effective transmission of the virus via *Polymyxa betae* and for virus assembly (Schmitt *et al*., 1992; Henry *et al*., 1995; Tamada *et al*., 1996).

In conclusion, in this work, natural biodiversity existing in wild beet species was characterized in the context of their resistance response against rhizomania disease. A precise real-time PCR-based quantification may have its impact on the duration of a selection cycle and on further studies concerning possible mechanisms, interactions and contributing factors involved in the development of resistance/tolerance. The procedures may for example be applied for the identification of a resistance target, i.e. virus vs. vector and for precise supplementation of plant disease descriptors in a genebank. In this study the rhizomania resistance score of analysed accessions was confirmed, apart from 45512 IDBB. However, taking into account specific features of wild beet reproduction, i.e. open- pollination and out- crossing, it would obviously be advisable to extend a representative group of individuals in such a case in order to unravel an expected variation existing within a given genetic pool of a particular accession. An interesting and unexplored issue constitute also possible viral interactions, especially in light of the typical for rhizomania disease phenomenon of co-occurrence of two or more different viruses. In highly resistant accessions analysed here the mechanisms directed against the virus and/or viral elements crucial for vector transmission/pathogenicity seem to be particularly active, as the content of *Polymyxa betae* in some of them was shown to be comparable to other, more resistant plants, whereas at the same time viral RNA components, depending on their major function, display differential regulation. Therefore, taking into account this as well as the lack of enhanced symptoms of cell death in the group of highly resistant accessions, it seems probable that the major outcome of the host response is determined by an interplay between silencing and suppressor mechanisms. Nevertheless, other stages of the infection cycle and influence of other resistance-breaking viral pathotypes should also be examined in light of the potential impact of different contributing factors and different conditions on resistance development. Finally yet importantly, the presence of some features that may be unique and indicative of highly resistant accessions was demonstrated,

including selected rhizomania resistance-segregating sequences, morphological features and HRM profile. Here sequence diversity in DNA encoding FBK, a known p25 partner, in a group of wild beets representing different resistance/susceptibility sources was confirmed for the first time, thus underlining its potential role for resistance development, which was also referred by us to the current state-of-the-art literature. The actual implications of these observations require further verification.

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