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Efficiency of *Rz1*-based rhizomania resistance and molecular studies on BNYVV isolates from sugar beet cultivation in Greece

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Abstract A survey was carried out to investigate the current situation concerning rhizomania disease incidence in sugar beet cultivation of Greece. A systematic field evaluation over locations and years revealed a consistent disease severity pattern according to favourable agroclimatic conditions and pointed to the so far effectiveness of the *Rz1* gene-based resistance, as no major disease outbreaks were observed. Molecular analyses aiming at the characterization of the type and genetic diversity of the virus further confirmed the widespread occurrence of BNYVV in the country, as evidenced by RT-PCR amplification of all five known genomic molecules and nested-PCR assays. None of the isolates contained an RNA 5, typically found in pathotype P. On the basis of RFLP patterns, all BNYVV isolates analysed were classified as pathotype A. Sequence determination of the full-length RNA 3-encoded p25 protein, responsible for symptom development, revealed amino acid motifs ACHG/VCHG in the hypervariable region $aa_{67–70}$. The presence of valine in position 67 did not appear associated with increased pathogenicity and resistance breaking properties, as earlier reported.

Keywords *Beet necrotic yellow vein virus* · *Rz1*-based resistance · RNA 3-encoded p25 protein · Rhizomania · Sugar beet

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Introduction

Rhizomania disease of sugar beet (*Beta vulgaris* subsp. *vulgaris*) was initially described by Canova in Italy in the mid 50's and has since been reported in most crop producing countries worldwide (Tamada 1999). The disease causes severe economic losses as a consequence of a dramatic reduction in root yield and sugar content. Rhizomania disease symptoms include massive lateral root proliferation resulting in a beard-like appearance, constriction of the main taproot and stunting of the infected plant (Tamada 1999). The disease is caused by *Beet necrotic yellow vein virus* (BNYVV) (Tamada and Baba 1973) which

is disseminated by the zoospores of the widespread soil-borne plasmodiophorid *Polymyxa betae* Keskin.

BNYVV is the type species of the genus *Benyvirus* (Torrance and Mayo 1997; Tamada 1999, ICTV: 00.088.0.01), having a rod-shaped particle and a multipartite positive single stranded RNA genome consisting of four genomic messenger-sense RNAs, with some isolates harbouring a fifth RNA molecule (Tamada et al. 1989). All genome segments have 5′-cap and a 3′-poly (A) tail (Putz et al. 1983). RNAs 1 and 2 are required for basic house-keeping functions including replication, packaging and cell-to-cell movement (Tamada 1999), whereas the natural infection process involves the host-specific function of additional proteins, directly involved in pathogenesis and vector transmission, encoded by the small RNAs (Richards and Tamada 1992; Tamada and Abe 1989; Tamada 1999).

Based on molecular characteristics, including RNA composition and nucleotide sequence divergence (Koenig et al. 1995), BNYVV has been classified in three major pathotypes designated as A, B and P. Type A is widespread in most EU countries, USA, China and Japan whereas the B type is more restricted and mainly found in Germany and France (Kruse et al. 1994). The third strain group, designated as pathotype P, contains an additional genomic RNA (RNA 5) and was found at first in the region around Pithiviers, France (Koenig et al. 1997), later in Kazakhstan (Koenig and Lennefors 2000) and more recently in the UK (Ward et al. 2007). Other RNA 5-containing isolates have been reported in Japan, China (Tamada et al. 1989; Kiguchi et al. 1996; Miyanishi et al. 1999), the UK (Harju et al. 2002; Ward et al. 2007) and Germany, where one field has been found to be infested by an RNA 5-containing East Asian isolate (Koenig et al. 2008). On the basis of sequence variability, isolates containing a fifth RNA molecule have been classified into P- and J-types for the European and Asian isolates respectively (Schirmer et al. 2005). Analyses employed for strain differentiation include single-strand conformation polymorphisms (SSCP), restriction fragment length polymorphism (RFLP) (Kruse et al. 1994; Suárez et al. 1999) and partial or complete sequencing combined with phylogenetic analysis that allows for the classification of BNYVV isolates with respect to pathogenicity, sequence diversity and geographic origin (Koenig and Lennefors 2000; Schirmer et al. 2005).

Given the aggressiveness of RNA 5-containing isolates (Tamada et al. 1996; Miyanishi et al. 1999), it has been proposed that RNA 5-encoded p26 acts in a synergistic manner with RNA 3-encoded p25 (Kiguchi et al. 1996; Heijbroek et al. 1999; Link et al. 2005), the latter being regarded as the main BNYVV pathogenicity factor (Tamada et al. 1999). Recent reports for severe rhizomania symptoms on cultivars harbouring the *Rz1* resistance gene, led to the hypothesis of resistance breaking by mutations resulting from high selective pressure to overcome this gene. High sequence variability of A-type BNYVV RNA 3-encoded p25, specifically at amino acids 67–70 ($aa_{67–70}$), has been associated with varying pathogenicity (Tamada et al. 2002; Schirmer et al. 2005; Chiba et al. 2008). In addition to $aa_{67–70}$, aa residues at positions 129, 135 and 179 have also been proposed as possible factors in variable pathogenicity and resistance breaking incidence (Acosta-Leal et al. 2008; Chiba et al. 2008; Koenig et al. 2009).

Rhizomania was initially observed in Greece in 1972, in the region of Larisa, and since then has rapidly spread throughout all crop growing zones in the country. To cope with the problem and provide effective protection to the crop, apart from other relevant agronomic practices (i.e. lengthy rotations, early sowing, avoidance of excessive irrigation etc), resistant varieties were sown as they became available. When BNYVV pathotypes were first studied in Europe, a single sample from the region of Larisa was found infected by pathotype A (Kruse et al. 1994).

Experience from past years, based on commercial sugar beet growing and related experimentation, pointed to a pronounced consistent pattern of rhizomania disease severity in the various main cultivation zones of the country: there is a general decrease from south to north, although fluctuations in severity symptoms and damage are occasionally encountered. Such a general trend appears associated with agroclimatic conditions that are more favourable for disease development towards the southern regions. Additionally, resistant varieties used throughout the country seem to effectively protect the crop against rhizomania. However, the long time elapsed since the identification of pathotype A in only one of all growing regions and, particularly in view of the findings on “resistance-breaking” in fields sown to varieties endowed with the *Rz1* gene (Liu et al. 2005; Schirmer et al. 2005; Rush et al. 2006; Acosta-Leal et

al. 2008; Chiba et al. 2008; Koenig et al. 2009), necessitated a thorough investigation of the situation currently evolved in the country. The objectives of this study were a) a detailed field evaluation of the efficiency of *Rz1* gene-based rhizomania resistance b) the molecular characterization of BNYVV isolates from representative fields in all five major sugar beet growing regions, in order to determine the type and genetic diversity of the virus and c) the determination of the amino acid composition of RNA3-encoded p25 protein of isolates collected from the above areas.

Materials and methods

Field performance evaluation

Twelve field experiments were conducted in the years 2004, 2005 and 2006, in four of the main regions of sugar beet cultivation in the country i.e. the areas of Larisa, Platy, Xanthi and Orestiada (Fig. 1). Experimentation in the fifth main growing region of Serres was not considered necessary due to its close proximity and similarity with the region of Platy. The experimental material all together consisted of a standard rhizomania susceptible commercial variety (SC), six imported rhizomania resistant commercial varieties (RC) and thirty experimental/pre-commercial hybrids (*Rz1*-Hyb). The susceptible variety (Alexandra), as well as the hybrids used, are products of the mainstream sugar beet local breeding program. The latter owed their resistance exclusively to the *Rz1* gene and were employed so that effectiveness of this gene could be assessed. The commercial resistant varieties most probably are also endowed with this gene. However, no concrete knowledge on the background genotype of these varieties is available as this is mostly non-disclosed information.

The experimental layout was that of a randomized complete block (RCB) design with six replications. Each experiment included the susceptible variety, 4 resistant commercial varieties and 15 resistant experimental/pre-commercial hybrids. The experimental plots were treated according to the standard agronomic practices of each region (fertilizer and water regimes, crop protection etc). Each experimental plot consisted of four rows, of which the two middle rows were harvested (7.4 m^2) to provide material for the measurements.

Fig. 1 The five major sugar beet growing zones in Greece (Orestiada, Xanthi, Serres, Larisa, Platy). The spots correspond to the areas where samples were collected

Variables measured were root fresh weight (tonne/ha), sucrose content (% of fresh root weight) and the content of the melassigenic compounds K^+, Na^+ and α-amino N (mg/100 g root weight). All measurements were performed in an automated VENEMA beet analyzer line. White sugar was calculated on the basis of gross sugar (the product of root weight and sucrose content) and the amount of melassigenic compounds, according to the widely used formula of Reinefeld (Reinefeld et al. 1974).

Data were analyzed through standard ANOVA procedures for all variables involved, using the statistical package JMP v.6.

Virus source plant material for molecular analyses

Sugar beet roots manifesting rhizomania symptoms were collected in year 2005 from 40 fields representatively covering each sugar beet growing area in Greece (Fig. 1). Samples were collected from various fields in the areas of Larisa, Xanthi, Platy and Orestiada, including the sites where field experiments were conducted, each site being an average representation of the respective area. For the region of Serres, samples originated from several commercial fields representing the entire area.

All samples tested came from a diploid, locally bred commercial variety, whose resistance is based on

the *Rz1* gene. The occurrence of the disease was verified in 38 sugar beet samples by means of DAS-ELISA (Biorad). Sugar beet root tissue infected with BNYVV pathotypes A, B, and P, supplied by Prof. M. Varrelmann (University of Göttingen, Germany) were used as reference material.

RT-PCR and nested-PCR amplification

Total RNA isolation was performed on taproots and rootlets with an RNeasy plant mini kit (Qiagen) and was subsequently reverse transcribed using the Im-Prom II Reverse Transcriptase System (Promega). A single universal 3′end primer, common for all BNYVV RNA molecules and four specific forward primers (Suárez et al. 1999) were used for the amplification of RNAs 1–4. The forward primer 5′-atcaagaacattttaccagaag-3′ and reverse primer 5′-cacatttcacatccagtcagta-3′, derived from the sequence with Accession No. D63936 (Kiguchi et al. 1996), were used for the specific amplification of BNYVV RNA 5. Four pairs of internal primers (Suárez et al. 1999) were used in the nested-PCR for RNAs 1–4. PCR and nested-PCR were performed using standard molecular techniques (Sambrook et al. 1989), at annealing temperatures 50°C and 55°C respectively.

RFLP analysis for the characterization of BNYVV pathotypes

Differentiation of isolates on the basis of RFLP patterns was based on the detection of the following cleavage sites: *Hinc*II for RNA 1 (6116–6574), *Taq*I, *Hinc*II and *Msp*I for RNA 2 (4159–4501), *Msp*I and *Bam*HI for RNA 3 (476–1734), *Hinc*II and *Taq*I for RNA 4 (668–1325). Digestions were performed as previously described by Kruse et al. (1994) and the derived restriction patterns were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide.

Nucleotide sequence variability of RNA 3-encoded p25

The primer pair 5′-tgatttagggcacagacctt-3′ (forward)/ 5′-catgatatgaggtttagcataacc-3′ (reverse) was used to amplify the full-length sequence of p25 from BNYVV-infected field grown sugar beet roots, originating from the different sugar beet growing Greek regions, by means of a two-step RT-PCR assay. PCR reactions were carried out using a high-fidelity DNA polymerase (Phusion High Fidelity DNA polymerase, Finnzymes), at annealing temperature 50°C. Amplification products were cloned and sequenced using M13 forward and reverse primers.

Results

Evaluation of *Rz1*-based rhizomania resistance under field conditions

As is well known, rhizomania reduces both root yield and sucrose content while at the same time it increases the content of melassigenic compounds K, Na, and amino-N (Rush 2003), thus lowering juice purity and consequently diminishing the end product i.e. ‘white or crystal sugar’. Experimental data collection and analysis pertained to all these variables. The composite variable white sugar however, a combined result of root yield, sucrose content and impurities in the root sap, forms the most simple and appropriate variable for evaluating field performance as it collectively reflects the effect of rhizomania on all above mentioned single variables.

The effectiveness of rhizomania resistance conferred by the *Rz1* gene was assessed based on the average performance of the five best experimental hybrids (*Rz1*-Hyb.-Average) in each experiment, owing their resistance exclusively to this gene, as related to that of the standard susceptible cultivar (SC) and of the four resistant commercial varieties in each experiment (RC-Average). The aforementioned averaging was considered as appropriate to avoid significant confounding with a variety’s productivity *per se* (i.e. regardless of the resistance trait). Also by using the five best *Rz1* hybrids, their average performance would be comparable to the average performance of the commercial resistant hybrids.

A summary of the results of all 12 experiments (Pavli, 2010) is shown in Table 1. Data presented refer to the white sugar production of the susceptible variety (SC), of the highest and lowest among the five best yielding *Rz1*-endowed experimental hybrids (H-*Rz1*-Hyb and L-*Rz1*-Hyb accordingly), and of the highest and lowest among the four resistant commercial varieties (H-RC and L-RC accordingly). The averaged performances, on the

Table 1 White sugar (tonne/ha) production of the susceptible variety (SC), the highest and lowest of the five best yielding *Rz1*-endowed experimental hybrids (H-*Rz1*-Hyb and L- *Rz1*-Hyb) and the highest and lowest of the four commercial resistant varieties (H-RC and L-RC) in all twelve experiments

Year	Varieties	Larisa	Platy	Xanthi	Orestiada
2004	SC	3,41	2,67	3,38	6,21
	H-*Rz1*-Hyb	7,68	9,84	10,84	11,88
	L-*Rz1*-Hyb	6,98	8,88	10,00	11,05
	H-RC	6,10	8,80	11,01	12,35
	L-RC	4,34	7,81	7,73	9,09
	LSD (0.5)	1,96	0,93	1,14	1,85
2005	SC	2,49	4,72	3,84	9,14
	H-*Rz1*-Hyb	9,18	11,89	13,87	15,56
	L-*Rz1*-Hyb	7,93	11,18	13,06	15,22
	H-RC	7,59	11,90	14,10	15,66
	L-RC	6,93	10,56	11,33	13,66
	LSD (0.5)	1,63	1,03	1,36	2,50
2006	SC	2,91	5,62	2,58	9,55
	H-*Rz1*-Hyb	6,48	9,64	10,50	13,20
	L-*Rz1*-Hyb	6,09	9,12	8,66	12,46
	H-RC	7,48	10,28	9,77	13,27
	L-RC	5,52	8,57	8,12	10,19
	LSD (0.5)	1,27	1,19	1,52	ns

basis of which effectiveness of rhizomania resistance was assessed as already mentioned, are presented in Fig. 2a, b, c. Differences observed between the commercial resistant cultivars and the resistant experimental hybrids although statistically significant in some cases, were indeed relatively small and proved altogether non-significant in 2006, when two better yielding commercial varieties were included in the experiments. More importantly, the two resistant categories presented the same trends in all locations and years. The fact that the commercial resistant hybrids yielded very similarly to the group of the best *Rz1*-experimental hybrids, substantiates the belief that the former base their resistance on at least the *Rz1* gene.

Based on long term cultivation and experimental results under rhizomania-free conditions—when and where such fields were still available—the susceptible variety Alexandra (grown since 1993) proved a very productive cultivar with a quite stable throughout-the-year performance (usually outyielding the commercial resistant cultivars by 5–10%). Such a performance was better in southern areas due to its late maturing (NZ-Z type) and cercospora leaf spot resistance characteristics. Consequently, it's decreasing white sugar yield from north to south when tested under rhizomania conditions can safely be attributed to the effect of rhizomania and thus points to an increase of disease severity in the same direction. The exception to this general trend however, was the area of Xanthi where this variety usually suffers losses from the disease similar to the ones in Larisa. At the same time, the performance of the experimental hybrids protected with the *Rz1* gene is consistently increasing from south to north, an increase though being less than expected in the region of Xanthi in year 2006 (Fig. 2, Graphs c, d).

PCR amplification of genomic RNAs and RFLP analysis of Greek BNYVV isolates

In 2005, the five major sugar beet production areas in Greece were monitored for the possible occurrence of rhizomania by visual inspection. In total, 40 samples of plants suspected to be infected were collected and of these 38 scored positive in ELISA for BNYVV (data not shown). The positive samples originated from all 5 production areas, indicating a widespread occurrence within Greece. All samples gave positive RT-PCR amplification results for each of the RNA molecules investigated: 1,209 bp for RNA1, 750 bp for RNA2, 1,263 bp for RNA3, and 976 bp for RNA4 (data not shown), thus confirming the presence of the virus. None of the isolates gave a corresponding to RNA5 product of 440 bp, suggesting the absence of pathotype P in Greece. The identity of amplification products was verified by using internal primers. The resulting nested-PCR products were of the expected size: 459 bp, 343 bp, 346 bp and 658 bp corresponding to each of the four RNAs (RNA 1 through 4, respectively).

RFLP analysis was conducted for further molecular analysis and differentiation of the BNYVV isolates collected throughout Greece. Restriction patterns obtained were in most cases identical to those obtained from pathotype A, used as reference material. Expected restriction patterns, based on the published sequences from gene banks, were obtained using the following endonucleases: *Hinc*II (A: 6264) for RNA1 (6116–6574) (Fig. 3a), *Taq*I (A: 4407), *Hinc*II and *Msp*I (A: no restriction site) for RNA 2

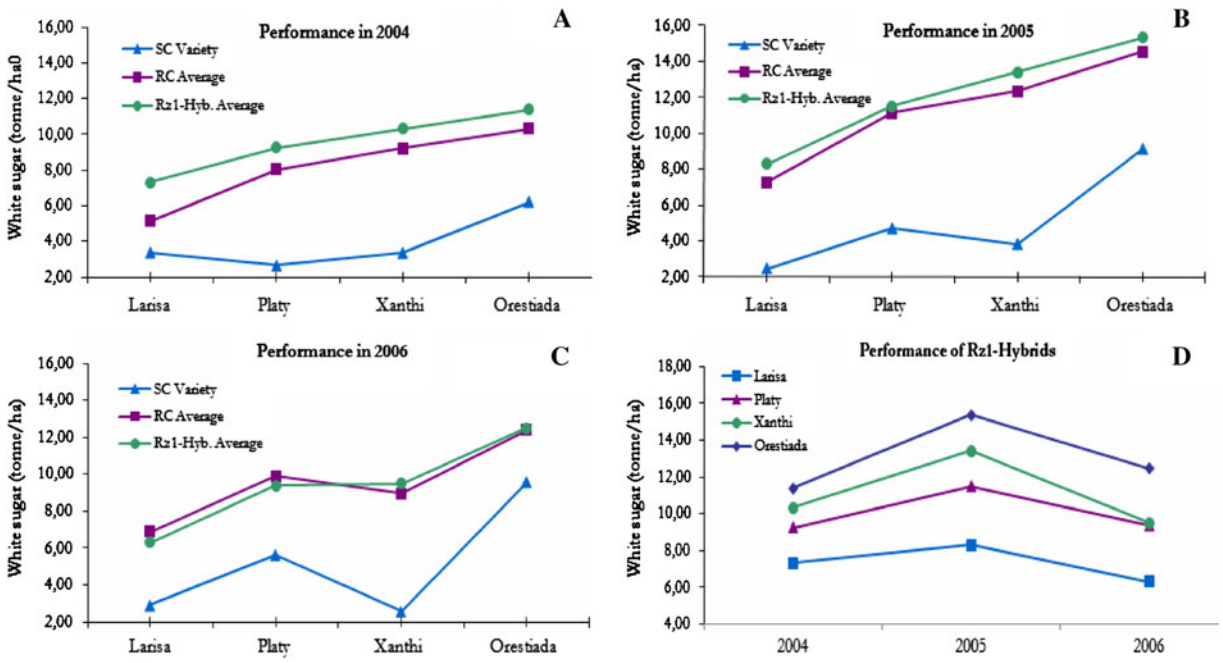

Fig. 2 Performance of the susceptible cultivar (SC), the average of the four resistant commercial varieties (RC-Average) and the average of the five best experimental hybrids, owing their resistance exclusively to the *Rz1* gene (*Rz1*-Hyb-Average) in four locations **a** in year 2004, **b** in year 2005, **c** in year 2006 and **d** the average performance of the five best experimental hybrids (*Rz1*-Hyb-Average) in four locations, in years 2004, 2005 and 2006

(4159–4501), *Msp*I (A: 1505; B: 799, 1505) and *Bam*HI (B: 752) for RNA 3 (472–1734) (Fig. 3b), *Hinc*II (A: 1105) and *Taq*I (B: 1157) for RNA 4 (668–1325) (Fig. 3c). The restriction patterns obtained with all endonucleases tested were for all 4 RNAs diagnostic for group A with the exception of two samples from neighbouring fields, presenting a polymorphism at the *Hinc*II restriction site of RNA 1. This polymorphism was further investigated by sequencing, which only revealed a single point mutation at this restriction site (data not shown). Digestion of the nested-PCR product of RNA 2 with *Hinc*II and *Msp*I could not differentiate pathotypes A and B.

Sequence determination of RNA 3-encoded p25 protein

The nucleotide sequences of amplicons corresponding to RNA 3-encoded p25 of different isolates, representing fields from all five beet growing areas, were determined and published in GenBank (Accession No. FJ224246, FJ224247, FJ224248, FJ224249, FJ224250). The percentages of sequence identities among the Greek isolates amount to 98–99%, both at the nucleic acid and amino acid levels. Comparisons from the sequences derived from the local isolates with previously published sequences of p25 have revealed a 99 % nucleotide sequence identity with the Italian isolate I12 (Table 2) (Accession No. AF197551) and 99% amino acid sequence homology with either Rsf or S5 isolate from the Imperial Valley, USA and Torralba Cva, Spain respectively (Accession No. AAU05673, AAU05679). The aa tetrad, proposed to be responsible for overcoming rhizomania resistance (Tamada et al. 2002; Schirmer et al. 2005; Chiba et al. 2008), of all Greek BNYVV isolates fell in groups A-ACHG and A-VCHG corresponding to A-type isolates lacking RNA-5 (Schirmer et al. 2005). Coding sequence for p25 aa 129, 135 and 179 had the motif $H_{129}D_{135}N_{179}$ in all samples analyzed.

Discussion

This study aimed at the investigation of current rhizomania disease incidence in Greece, based on

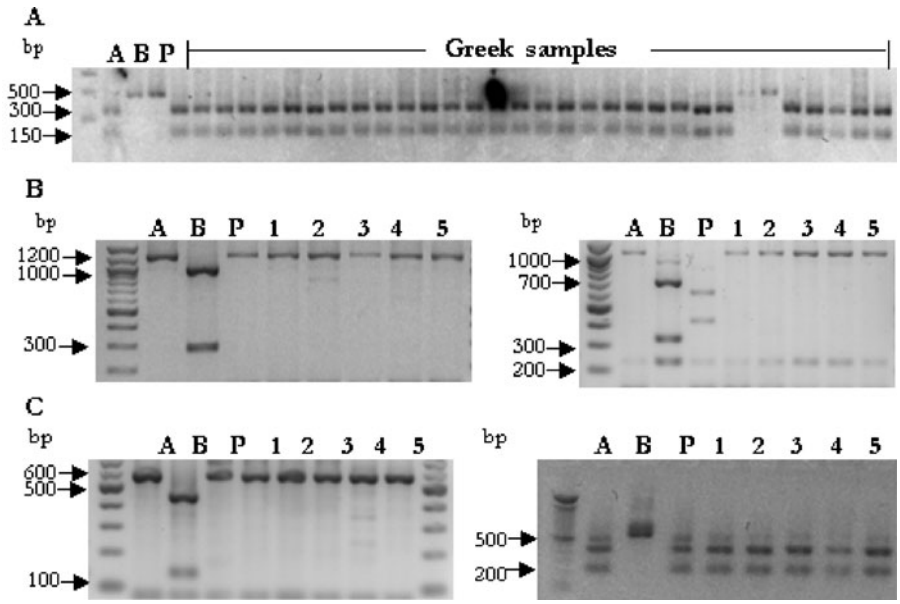

Fig. 3 RFLP patterns obtained with the RT-PCR products of the BNYVV isolates collected from various geographical regions in Greece. **a** RNA 1 (nt 6116–6574) digestion with *Hinc*II, **b** RNA 3 (nt 472–1734) digestion with *Bam*HI (*left*) and *Msp*I (right) and **c** RNA 4 (nt 668–1325) digestion with *Taq*I (*left*) and *Hinc*II (*right*). Lanes 1, 2, 3, 4, 5 correspond to samples from the five sugar beet growing areas and pathotypes A, B and P are used as a reference material. Ladder used: GeneRuler DNA Ladder Mix, Fermentas

field evaluation of susceptible and resistant cultivars as well as on molecular analyses for a general characterization of BNYVV isolates and particularly of the RNA 3-encoded p25, whose composition is determinant for strain virulence. The resulting information should be significant for the choice of imported resistant varieties as well as for the objectives of the local breeding program towards

Table 2 Comparison of the hypervariable amino acid residues at position 67–70, 129, 135 and 179 of *Beet necrotic yellow vein virus* RNA 3-encoded p25 of Greek (Gr1-Gr5), Turkish (T1, T9), Kazakh (Kas2), Italian (I12), Spanish (S5, E12) and American (Rsf) isolates

Location	Isolate	p25 motif (67–70, 129, 135, 179)							Accession N.(nt)	Reference
		67	68	69	70	129	135	179		
Xanthi	Gr1	V	C	H	G	H	D	N	FJ224246	This study
Serres	Gr2	A	C	H	G	H	D	N	FJ224247	This study
Larisa	Gr3	A	C	H	G	H	D	N	FJ224248	This study
Orestiada	Gr4	V	C	H	G	H	D	N	FJ224249	This study
Platy	Gr5	V	C	H	G	H	D	N	FJ224250	This study
Turkey	T1	A	H	H	G	H	D	D	AY772230	Kutluk Yilmaz et al. 2007
Turkey	T9	A	C	H	G	H	D	D	AY772232	Kutluk Yilmaz et al. 2007
Kazakhstan	Kas2	A	L	H	G	H	D	N	AF197553	Koenig and Lennefors 2000
Italy	I12	A	L	H	G	H	D	N	AF197551	Koenig and Lennefors 2000
Spain	S5	V	C	H	G	H	D	N	AY696171	Schirmer et al. 2005
Spain	E12	V	C	H	G	Y	D	N	EU330455	Koenig et al. 2009
USA (IV)	Rsf	V	C	H	G	H	D	N	AY696165	Schirmer et al. 2005
USA(IV)	Tae05	V	C	H	G	H	E	N	EU480513	Acosta-Leal et al. 2008

producing varieties with improved and durable resistance to the rhizomania disease.

The overall findings verify the widespread occurrence of rhizomania in all sugar beet production areas in Greece and further indicate that damage caused by the disease in the various growing regions is related to their geographic locations. As can be deduced from Fig. 2, white sugar yield of the susceptible variety expressed as percent of the average of the five best *Rz1*-hybrids ranged from 32–45, 30–60, 27–40 and 55–80 in the regions of Larisa, Platy, Xanthi and Orestiada respectively. In good agreement with previous empirical observations from the commercial cultivation, the results revealed a consistent pattern of disease severity increase from north to south, a direction in which more favourable conditions (warmer temperatures, heavier irrigation, earlier infestations) are prevailing. The exception in the area of Xanthi, where disease severity is similar to that of the southernmost Larisa area, definitely reflects to the greatest degree the effect of a favourable for the disease microclimate in this region. However, the lower yield of this variety in the specific area is also due, although to a much lower degree, to a variety-location interaction effect, as has been observed in the past under no disease conditions as well.

The consistency in yield increase of the *Rz1*-experimental hybrids—as well as of the resistant imported varieties—from south to north, substantiates the conclusion that rhizomania resistance due to the *Rz1* gene is quite stable for the time being. Based on the general trend established, the relatively lower than expected yield performance of the *Rz1*-hybrids and of the commercial hybrids in the area of Xanthi in the year 2006, could well be the result of a year effect, although other possible causes should not be excluded as discussed later.

Despite the general field stability of *Rz1*-resistance established by the present study, and the fact that no outbreaks have occurred so far, the absence of sufficient and current information on the virus molecular characteristics dictated the need for relevant studies. In this framework, RT-PCR targeting the five known BNYVV genomic molecules and nested-PCR analyses confirmed the presence of BNYVV in all ELISA-positive samples tested and further indicated the absence of pathotype P in the country, as evidenced by the absence of RNA 5-containing isolates. On the basis of the RFLP patterns obtained, in comparison to those of reference material, all BNYVV isolates analysed were classified as pathotype A. This is true despite of a minor polymorphism revealed by RFLP, a polymorphism not significant however for a change in classification.

Earlier studies on pathogenicity and ability of BNYVV isolates to overcome *Rz1*-based resistance, point to a possible involvement of certain amino acid residues of p25 and particularly those in positions 67–70, 129, 135 and 179 (Tamada et al. 2002; Schirmer et al. 2005; Acosta-Leal et al. 2008; Chiba et al. 2008; Koenig et al. 2009). Although a significantly higher variation occurs at amino acid residues 67–70 of p25 -$aa_{67–70}$, referred to as "tetrad"-, especially within BNYVV A-type (Schirmer et al. 2005), evidence for the importance of a drift resulting from positive selection to overcome resistance has so far been obtained only for valine at position 67 (Koenig et al. 2009). Such finding could explain the previously observed *Rz1*-resistance breaking ability of A-type isolates from Spain and the USA (Schirmer et al. 2005; Pferdmenges et al. 2009).

Analysis of the hypervariable amino acid sequence of p25 revealed that the Greek isolates contain ACHG and VCHG tetrad motifs in the areas of Serres, Larisa and Xanthi, Orestiada, Platy respectively. In this study however, the presence of V_{67}-possessing BNYVV isolates does not appear associated with disease outbreaks, as these have not occurred at least so far. At the same time, the V_{67} motif was not always accompanied by a high disease severity, as it was found in the northernmost area of Orestiada (Gr4) that consistently manifests the lowest disease severity in the country. To the contrary, the A_{67} motif was present in the southernmost Larisa area (Gr3), characterized by a high disease severity, whereas in the areas of Platy (Gr5) and Serres (Gr2) with similar climatic conditions and disease development, different motifs were observed.

Similar findings have been reported by Liu and Lewellen (2007) who describe V_{67}-possessing BNYVV isolates with no resistance breaking abilities, as opposed to isolates with differing aa_{67} residues which were able to cause severe damage to partially resistant cultivars. However, particularly in the light of the solid findings of Koenig et al. (2009), it seems logical to assume that no resistance breaking has occurred to date because the V_{67} motifs observed form part, not yet positively selected for, of a large genetic variability of the pathogen populations present

in the fields. If this is a plausible explanation, then the relatively lower than expected yield of the resistant *Rz1*-hybrids and cultivars in Xanthi in the year 2006 deserves special attention and requires further investigation as it might reflect a building of a higher concentration of V_{67}-possessing isolates already identified in the area. In this case, such an evolution could eventually result in resistance breaking phenomena as observed elsewhere.

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