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RESEARCH ARTICLE

M. Belén Suárez[1]
Isabel Grondona[1]
Pablo García-Benavides[2]
Enrique Monte[1]
Isabel García-Acha[1]

[1]Department of Microbiology and Genetics, CSIC–University of Salamanca, Spain
[2]Regional Diagnostic Center, Junta de Castilla y León, Aldearrubia, Salamanca, Spain

Characterization of beet necrotic yellow vein furovirus from Spanish sugar beets

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Correspondence to:
Isabel García-Acha. Department of Microbiology and Genetics. CSIC–University of Salamanca.
Av. del Campo Charro, s/n.
37007 Salamanca. Spain.
Tel.: +34-923252092. Fax: +34-923224876.
E-mail: igarcha@gugu.usal.es

Summary Rhizomania is a viral disease, caused by beet necrotic yellow vein furovirus (BNYVV), which was detected in Spanish sugar beets in 1988, it being focused on the Castilla y León region. BNYVV has five RNA fragments with specific functions, and the different composition and proportion of RNA in the virions allow their separation and the characterization of their activities during the development of the disease. Thirty–six samples of sugar beet rootlets and frozen pulps from three different sugar beet zones of Castilla y León were analyzed by DAS-ELISA and Immunocapture-Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR) using specific primers. The identity of the cDNA products was confirmed by nested-PCR and restriction fragment length polymorphism (RFLP). The uniformity of the patterns obtained by RFLP analyses with nine endonucleases showed the existence of a unique strain of BNYVV in 80,000 Ha of crop surface which could be explained by a recent arrival of the rhizomania disease to this region. The isolates studied were more similar to type A, which has been previously described in BNYVV, but a non-expected cleavage site for this molecular group was observed with endonuclease *Hin*cII on the RNA-2 IC-RT-PCR product (nt 2133–3293) in the thirty–six Spanish samples and also in a North American strain taken as reference. The use of frozen pulps obtained as a previous step to the industrial extraction of sugar avoids problems due to erratic distribution of the virus in the roots, provides repetitive results for a particular sample, and facilitates epidemiological and distributional studies on rhizomania disease.

Key words Beet necrotic yellow vein furovirus (BNYVV) · Sugar beet · Rhizomania · Immunocapture · Reverse Transcription-PCR

Introduction

Sugar beet diseases have been catalogued [1, 26, 34, 36] on the basis of their viral, bacterial or fungal origin. Among viral diseases, rhizomania causes most economic losses. Initially described by Canova [7], who first attributed this disease to the association of the beet necrotic yellow vein furovirus (BNYVV) with a vector fungus (*Polymyxa betae* Keskin), rhizomania has been detected around the world in most geographical areas where sugar beet is cultivated. During the forty years since rhizomania was described, this disease has been found in most of the sugar beet regions in Europe (Table 1). Characteristic symptoms of rhizomania are the atrophy of the primary root, sometimes rotten, and proliferation of small lateral rootlets (beards), constriction of the root at the surface of soil, brown vascular rings and yellowing leaves [21, 23, 28, 36]. Only occasionally do the yellow veins which gave the name to BNYVV appear [32]. The term “rhizomania” refers to the root bearding where most of the virus concentrates.

The traditional diagnosis of BNYVV is based on inoculation of indicative plants such as *Chenopodium quinoa* or on the study of symptoms of the diseased plant and immunological tests such as double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Since symptoms may be very similar to those produced by the attack of several pathogenic worms, and the fact that serological cross-reactivity with other viruses present in sugar beet was reported [37], molecular PCR-based techniques have been developed for detection of BNYVV [16, 27, 38].

BNYVV is a furovirus [33] with a series of peculiarities [25]. At least five types of virions are known, all of them

Table 1 Detection of rhizomania disease in Europe

Year	Country	Author(s)
1950	Italy	Canova
1973	Greece	Kouyeas
1974	France	Putz and Vuittenez
1977	Germany	Hamdorf et al.
1978	Yugoslavia	Sutic and Milanovic
1981	Austria	Krexner
1981	Rumania	Codrescu et al.
1983	Switzerland	Haeni and Bovey
1984	The Netherlands	Heijbrook
1984	Czeck Republic	Novak and Lanzova
1984	Bulgaria	Jankulova
1986	Sweden	Lindsten
1987	Belgium	Verhoyen et al.
1987	United Kingdom	Asher
1988	Spain	Raposo and Mateo Sagasta
1988	Spain	Cambra and García Benavides
1989	Hungary	Horvath et al.

helicoidal and rigid [30], containing a molecule of RNA and several copies of a 21 kDa protein [20] which forms the capsid. BNYVV differs from other furoviruses in that it is divided into five fragments of RNA [9] with specific functions, all of them possessing poly(A) sequences at the 3´ end. Only RNAs 1 and 2 are required for leaf infections and RNAs 3, 4 and 5 may disappear spontaneously from BNYVV isolates after passage on leaves. The different composition and proportion of RNA in the virions allow their separation and the characterization of their activities during the development of the disease. Differences in RNA 3, 4 and 5 contents are often associated with distinct local lesion symptom types [25]. RNA fragments of an array of BNYVV isolates have been studied [15, 17, 18, 29, 39] and the genomes of two isolates of this virus have been completely sequenced: F2 [3–5, 24] and S [29], the nucleotide sequence divergence between them being scarce [29].

In molecular approaches using restriction fragment length polymorfism (RFLP), two major strain groups, named type A and type B, were described within BNYVV. The four Spanish isolates studied fell into group A [17].

In Spain, rhizomania was simultaneously described in two separate publications [6, 22] demonstrating the presence of this disease in Castilla y León (North-West of Spain). Later on, rhizomania was also detected in other sugar beet zones (Center of Spain) [12]. However, there is no evidence that the typical rhizomania disease has extended to the autumn sugar beet in Andalucía (South of Spain).

In the present study, we have analyzed by immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) [19] thirty–six samples of spring sugar beet from North-Western Spain that had tested positive in DAS-ELISA for rhizomania, using specific primers [31] for the four fundamental RNAs of the genome of this virus. RFLP profiles of IC-RT-PCR products were used to compare isolates among themselves and to a reference strain of BNYVV.

Materials and methods

Viruses The thirty–six samples included in this study came directly from rootlets or frozen pulp of sugar beet and were collected in three different areas (twelve samples per area) of Castilla y León: León (North), Palencia and Valladolid (Center), and Salamanca, Ávila and Segovia (South). Samples were analyzed by the DAS-ELISA test for rhizomania and also by IC-RT-PCR. Reference BNYVV strain CO94-4 from USA (supplied by Drs. B.R. Lovic and C.M. Rush, Amarillo, Texas, USA) was maintained in *C. quinoa* leaves frozen in glycerol at –20°C.

ELISA The protocol used was a double-antibody sandwich procedure [8] using BNYVV antibodies (Boehringer Mannheim, Mannheim, Germany). Absorbance values greater than 2x the mean of the negative control values were considered positive.

Immunocapture-Reverse Transcription Sugar beet juice for subsequent use on the IC Microtiter plates was extracted using a manual system: homogenization in a mortar of 1 g of sample in 5 ml of extraction buffer (0.5 M Tris HCl, pH 8.3, 150 mM NaCl, 0.05% Tween-20, 2% polyvinylpyrrolydone-40, 1% polyethylenglycol-6000 and 3 mM sodium azide).

Plates with a v-shaped end were coated with 50 µl per well of BNYVV antibodies solution (Boehringer Mannheim, Mannheim, Germany) at the calculated optimum concentration, generally 1/100 in carbonate buffer, pH 9.6. The plates were covered with a lid and after 2 h incubation at 37°C, the wells were washed with PBS-Tween buffer (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 0.05% Tween-20). Then, 50 µl of plant extract was added to each well. The plates were covered again and incubated overnight at 4°C. After washing three times with PBS-Tween buffer, 20 µl of reverse transcription (RT) mixture was added to each well. The RT reaction was performed according to the method of Nolasco et al. [19] with modifications. The RT reaction mixture contained 5 mM $MgCl_2$, 1 mM of each dNTP, 20 units of ribonuclease inhibitor, 15 units of AMV reverse transcriptase (Promega, Madison, USA), 2 µl of 10x reverse transcription buffer administered with the enzyme, 0.5 µl of oligo(dT) primer and water to a final volume of 20 µl. The plates, previously covered with a lid to avoid evaporation, were incubated at 37°C. After 3 hours the solution in each well was transferred to Eppendorf tubes and heated at 90°C for 2 min to stop the reaction. Then 15 µl were used in the PCR reaction.

Design of primers One downstream universal primer, at the 3´ end, common to the four RNAs under study, and four upstream specific primers (primers 1, 2, 3 and 4) were selected, based on the BNYVV-F2 sequences previously published [3–5]. Likewise, four pairs of nested primers (5 and 6, 7 and 8, 9 and

Table 2 Primers used for Immunocapture-Reverse Transcription-PCR (IC-RT-PCR) and nested-PCR amplification of Beet Necrotic Yellow Vein Furovirus (BNYVV) RNAs

Primer	Sequence*	Nucleotide position on RNA
Universal	5′-TTC ACA CCC AGT CAG TAC A	6704/RNA1; 5574/RNA2 1735/RNA3; 1330/RNA4
1	5′-ATG GTC TAA GGA GGC ACA T	5496/RNA1
2	5′-AAG CAT GTA GCC GAG TCC AT	3825/RNA2
3	5′-TGA TTT AGG GCA CAG ACC TT	473/RNA3
4	5′-ACT GCT AGG ATG GTG CAG AA	355/RNA4
5	5′-CGA AGA TAG CAG CAC ACA GGT TC	6116/RNA1
5	5′-TCA AGA TAG GAG GCC TGT GGC AT	6574/RNA1
7	5′-CGC GGT GTT TGT TGA ATA TCG TG	4162/RNA2
8	5′-CTT CGG AAC AAC CCA ATA GGA G	4504/RNA2
9	5′-CAC ATG TGA TGA TTG TAG CCT GTG	1335/RNA3
10	5′-CAT GAT ATG AGG TTT AGC ATA ACC	1679/RNA3
11	5′-GGT ATA TTC CAT GGA TGG CAG G	574/RNA4
12	5′-CTT ACC ATA GCA AGG AGG CTT G	1231/RNA4

*Derived from BNYVV F2 sequences published by Bouzoubaa et al. [3–5]

10, 11 and 12) were also designed on the sequence of RNA-1, RNA-2, RNA-3 and RNA-4, respectively. The four primer pairs described by Kruse et al. [17] were also used to characterize our BNYVV isolates. All primers were synthesized in an Applied Biosystems Model 391 PCR-Mate DNA synthesizer. Primer sequences and nucleotide position in each RNA are shown in Table 2.

PCR cDNA was amplified using the following pairs of primers: universal and 1, universal and 2, universal and 3, and universal and 4. The PCRs were carried out independently in order to facilitate the reading and interpretation of the results; they can be performed on the same IC plate if a suitable thermocycler is available or by collecting the cDNA of each well and transferring it to Eppendorf tubes. For PCR, 85 µl of the following amplification mixture were added to each tube: 1.5 mM $MgCl_2$, 500 ng of each primer, 0.2 mM of each dNTP, 2.5 units of *Tfl* thermostable DNA polymerase (Epicentre, Madison, USA), plus 5 µl of 20x reaction buffer administered with the enzyme. The solutions were overlaid with mineral oil and subjected to 35 cycles of 94°C for 1.5 min, 51°C for 1.5 min, 72°C for 1.5 min, and maintained at 4°C after the final cycle. No extension steps were needed during the first and last cycles.

The PCR products were analyzed by electrophoresis in gel of 1.7% agarose in Tris Borate-EDTA buffer, using ϕX 174/*Hae*III Markers (Promega, Madison, USA) as DNA ladder. Each lane was loaded with 10 µl of each PCR reaction mixture. The gel was run for 90 min at 100 V, stained with ethidium bromide, and examined under ultraviolet light.

Nested-PCR The identity of the IC-RT-PCR products with BNYVV genomic RNA fragments was checked with a nested-PCR. Each of the four PCR products, obtained with the outer primer pairs, was extracted by means of the Gene Clean Kit (Biosis 101, La Jolla, USA) and used as substrate for a second PCR with the nested primer pairs. The amplification mixture and the PCR conditions were similar to those of the first reaction, but the annealing temperature was increased to 62°C.

Characterization of BNYVV isolates by RFLP analyses IC-RT-PCR products were digested with restriction enzymes: *Msp*I, *Pvu*II, and *Bst*UI for RNA-1 (nt 5496–6704); *Taq*I, *Xba*I and *Bst*UI for RNA-2 (nt 3825–4574); *Taq*I, *Msp*I, *Bst*UI, and *Hin*cII for RNA-3 (nt 473–1735); and *Apa*LI, *Sca*I, *Taq*I, and *Hin*cII for RNA-4 (nt 355–1330). Restriction patterns of these PCR products were analyzed by electrophoresis following the conditions described above. Further characterization was carried out using the primer pairs and PCR conditions described by Kruse et al. [17]. In order to detect the existence of cleavage sites and determine the relationship of our BNYVV isolates to the major strain groups, the following restriction enzymes were used: *Sca*I and *Eco*RI for RNA-1 (nt 19–1088); *Bst*UI, *Taq*I and *Hin*cII for RNA-2 (nt 2133–3293); *Eco*RI and *Msp*I for RNA-3 (nt 50–1268); and *Apa*LI and *Hin*cII for RNA-4 (nt 87–1301).

Results

Extracts from all samples gave a positive result in DAS-ELISA test for rhizomania. In the analyses by IC-RT-PCR, the immunocapture of BNYVV allowed us to carry out the RT reaction without needing to extract or purify the viral RNA by alternative physical and/or chemical treatments [19], the time of preparation and analysis of the sample being reduced to only 24 h. No differences were observed between the size of PCR products obtained from the thirty–six samples of sugar beet rootlets and pulps, and the amplicons obtained from leaves of *C. quinoa* inoculated with the American BNYVV CO94-4 (Fig. 1). Through independent reactions, a PCR product of the expected size was obtained for each of the four RNAs

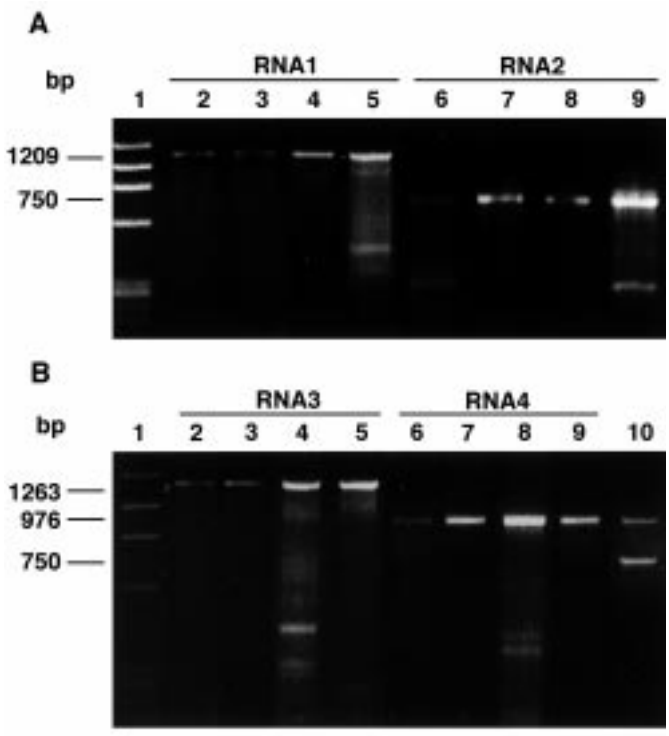

Fig. 1 Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) products from extracts of rhizomania-diseased sugar beets (A: lanes 2–4 and 6–8. B: lanes 2–4, 6–8 and 10) and beet necrotic yellow vein furovirus (BNYVV)-inoculated *Chenopodium quinoa* leaves (A and B: lanes 5 and 9), using the BNYVV specific primer pairs. A: universal and 1 (lanes 2–5) and universal and 2 (lanes 6–9). B: universal and 3 (lanes 2–5), universal and 4 (lanes 6–9) and primers universal, 1, 2, 3 and 4 simultaneously in the same reaction (lane 10). In both A and B lanes, 2 and 6 correspond to sugar beet rootlets from Northern Castilla y León, lanes 3 and 7 to frozen pulps from Central Castilla y León, and lanes 4 and 8 to sugar beet rootlets from Southern Castilla y León (see text for details). Lane 1, ϕX174/*Hae*III DNA-ladder (250 ng total DNA)

investigated: 1209 bp with the pair of primers universal and 1, 750 bp with the pair universal and 2, 1263 bp with the pair universal and 3, and 976 bp with the pair universal and 4. Independent reactions with the universal primer and each of the RNA-specific primers offered clearer results than the four specific primers and the universal one mixed in a same PCR. In this case the bands corresponding to RNA-1 and RNA-3 did not usually appear (Fig. 1B, lane 10).

To confirm that IC-RT-PCR products corresponded to BNYVV, nested-PCR was carried out using internal primers (Table 2). In this second PCR, the expected sizes of the bands were, respectively, 459 bp, 343 bp, 345 bp and 658 bp, for the PCR products corresponding to RNA-1, RNA-2, RNA-3 and RNA-4 of BNYVV (Fig. 2, lanes 2–5).

BNYVV isolates studied were characterized through RFLP analyses. The restriction patterns obtained were repetitive for all IC-RT-PCR products from the thirty–six Spanish isolates and identical to those of the American BNYVV (data not shown). Expected fragments, based on the sequences of BNYVV-F2 [3–5], were obtained with the following restriction enzymes: *Pvu*II (6211) on RNA-1 (nt 5496–6704); *Xba*I (3948), *Taq*I (4078 and 4410) and *Bst*UI (739) on RNA-3 (nt 473–1735); *Sca*I (759) and *Taq*I (1157) on RNA-4 (nt 355–1330). However, *Bst*UI and *Apa*LI did not cut RNA-1 and RNA-4 respectively, and non-expected restriction patterns were observed with *Msp*I on both RNA-1 and RNA-3; *Bst*UI on RNA-2; and *Taq*I on RNA-3. *Hin*cII should not have cut RNA-4 (nt 355–1330) but one restriction site was detected. Based on the sequence of BNYVV-S [29], *Taq*I on RNA-2 and *Hin*cII on RNA-3, did not show the expected cleavage sites.

Further characterization was carried out using the primers and endonucleases described in order to differentiate between types A and B in BNYVV [17]. IC-RT-PCR products of the expected size were obtained in all samples. The restriction patterns observed fell in group A for RNA-1 (nt 19–1088) with *Sca*I and *Eco*RI; for RNA-3 (nt 50–1268) with *Eco*RI and *Msp*I; and for RNA-4 (nt 87–1301) with *Apa*LI and *Hin*cII. The RFLP profiles obtained for RNA-2 (nt 2133–3293) with *Taq*I, *Bst*UI and *Hin*cII are shown in Fig. 3. Based on the sequence of BNYVV-Yu2 (type A) [17] expected fragments were obtained on RNA-2 (nt 2133–3293) with *Taq*I and *Bst*UI. No cleavage products were expected with *Hin*cII, however restriction fragments of aproximately 1025 and 135 bp were observed in the thirty–six Spanish samples and also in the American strain considered as reference.

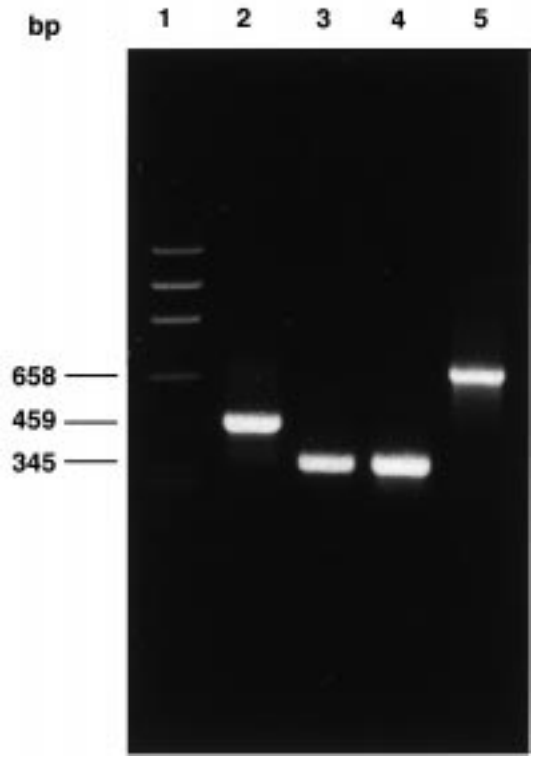

Fig. 2 PCR products using specific nested primers to beet necrotic yellow vein furovirus (BNYVV). Lanes 2–5, cDNA amplified using the primer pairs 5–6, 7–8, 9–10 and 11–12, respectively. Lane 1, ϕX174/*Hae*III DNA-ladder (250 ng total DNA)

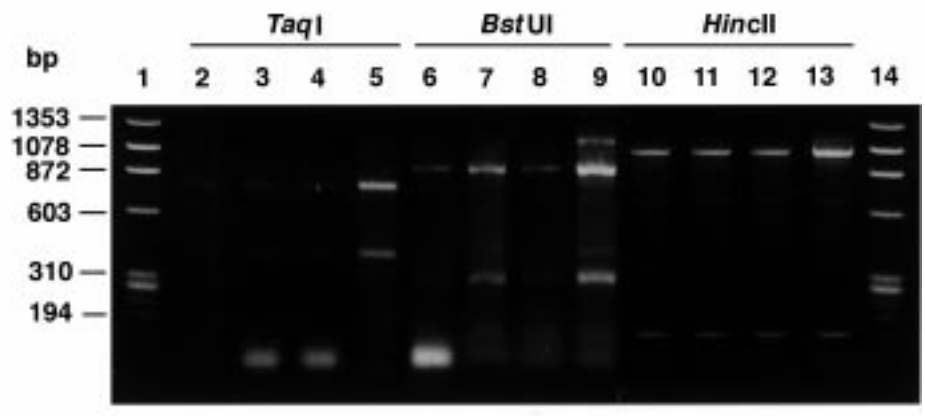

Fig. 3 RFLP patterns obtained with immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) products of beet necrotic yellow vein furovirus (BNYVV) RNA-2 (1160 nt: 2133–3293). Lanes 2, 6 and 10 correspond to sugar beet rootlets from Northern Castilla y León, lanes 3, 7 and 11 to frozen pulps from Central Castilla y León, lanes 4, 8 and 12 to sugar beet rootlets from Southern Castilla y León, and lanes 5, 9 and 13 to BNYVV-inoculated *Chenopodium quinoa* leaves. Lanes 1 and 14, ϕX174/*Hae*III DNA-ladder (250 ng total DNA)

Discussion

The sizes of the IC-RT-PCR and nested-PCR products confirm the existence of BNYVV in the naturally infected sugar beets analyzed and also in the BNYVV-inoculated leaves of *C. quinoa*. It was observed that during propagation on leaves, RNA-3 and RNA-4 can undergo frequent spontaneous deletions in experimental hosts. However, no differences in the size of the PCR products from naturally infected samples and *C. quinoa* leaves were observed.

The possibility, discovered by Nolasco et al. [19], of carrying out the reverse transcription of the RNA directly on the immunocaptured virus, without need for thermal or chemical disruption of the capsid of the virions [14, 35], means a great practical advantage for large scale diagnosis of diverse viral diseases especially of agricultural interest. We have tested infected sugar beet rootlets and frozen pulps by IC-RT-PCR. The pulps are obtained as a previous step to the industrial extraction of sugar, and pulp aliquots are kept frozen until subsequent analysis of their sugar content. The use of these pulps for detection of BNYVV by IC-RT-PCR avoids problems due to erratic distribution of the virus in the roots and provides repetitive results for a particular sample. The fact that great number of frozen pulps from different geographical origins are stored at sugar refineries facilitates epidemiological and distributional studies on rhizomania disease.

IC-RT-PCR needs careful optimization since the quality of the microtiter plate or the shape of the wells can affect the yield of immunocaptured viruses and the reproducibility of the results obtained with this technique. The best results were obtained with v-shaped end microtiter plates, and by carrying out the PCR in four independent reactions, but problems can arise particularly with RNA-1. When carrying out gel electrophoresis, the band corresponding to the PCR product using the pair of primers universal and 1 usually has a lower intensity than those corresponding to the other BNYVV RNAs. We consider that this may be due to a lesser accessibility of the RT to the RNA-1 in the immunocaptured virions, which would lead to a lower amount of the corresponding cDNA in the mixture. Alternatively it may be due to its greater molecular size with respect to the other RNAs. In contrast, the RNA-4 PCR product was the most suitable tool to detect BNYVV in natural samples since it can be more readily obtained than the others.

Our universal primer hybridizes with RNA-1, RNA-2, RNA-3, RNA-4 and RNA-5 of the BNYVV. We have also found a possible hybridization of this primer with the genome (RNA-1) of tomato black ring nepovirus (TBRV) which can attack sugar beet, destroying the main root and giving a tusk aspect to the secondary roots. However, TBRV is a virus with isometric particles (not a furovirus) with two genomic RNAs, both with poly(A) tails, and it is not immunocaptured by the antibodies used.

RFLP patterns obtained with all endonucleases tested, were identical in all samples analyzed. Results show the distribution of a single, unique strain of BNYVV in 80,000 Ha of crop surface in three different areas of Castilla y León which could be explained by a recent arrival of the rhizomania disease to this region. Since spontaneous mutations are reputed to be much more frequent among RNA viruses [10, 13], their populations consist of a large number of variants. However, the apparent genetic stability of Spanish populations of BNYVV studied is more in agreement with that found in some other plant RNA viruses such as the turnip yellow mosaic tymovirus [2] or the tobacco mild green mosaic tobamovirus [11].

The North-West Spanish population of BNYVV and the American strain studied are closer to isolate S than to F2 since their RFLP profiles were different from those expected for isolate F2 when *Msp*I on RNA-1; *Bst*UI on RNA-2; *Msp*I and *Taq*I on RNA-3; and *Hin*cII, and *Apa*LI on RNA-4 were used. However, cleavage site divergences with *Taq*I on RNA-2 and with *Hin*cII on RNA-3 were only detected when profiles were compared with those expected for BNYVV-S.

Considering the RFLP analysis described for detecting molecular types within BNYVV, all BNYVV isolates of Castilla y León studied in the present work belong to type A since most of their RFLP patterns are identical to those expected for this molecular group previously described as prevalent in Spain [16, 17]. However, we have observed an odd cleavage site with *Hin*cII

on RNA-2 (nt 2133–3293) which is absent in BNYVV-Yu2, an isolate considered as representative of type A, but also present in BNYVV-F2 (type B) and BNYVV-S (type A). This feature is present in all samples studied and could be considered as a proof of genetic stability of Spanish populations of BNYVV.

Since environment, crop conditions and rhizomania disease symptoms are different in sugar beets from Central Spain, new populations of BNYVV could be present. Further studies are needed in this part of Spain where rhizomania damages are not so important.

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