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Occurrence and incidence of viruses infecting green beans in south-eastern Spain

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Abstract A study was conducted to determine the identity and prevalence of viruses in 455 greenhouses in the main Spanish green bean growing area. Directed surveys were conducted in 422 crops from 2000–2004 to collect samples from diseased plants displaying symptoms that could be attributed to viruses. The samples were analysed to detect any virus by means of dsRNA extraction, mechanical inoculation to test plants, as well as ELISA and/or RT-PCR tests to detect potyviruses, geminiviruses and viruses previously known to infect beans in Spain. Random surveys were conducted in the years 2002 and 2005 (in 21 and 12 greenhouses, respectively) to study the actual incidence of known viruses in the area. Symptoms were recorded in 23,108 plants from which 664 plants were collected and analysed by ELISA or RT-PCR. The results of the directed surveys showed that all the analyzed crops carried the cryptic virus *Phaseolus vulgaris endornavirus* (PVuV), whereas phytopathogenic viruses appeared in smaller percentages of the crops: *Tomato yellow leaf curl virus* (TYLCV) 20.4%, *Southern bean mosaic virus* (SBMV) 9.0%, *Tomato spotted wilt virus* (TSWV) 4.0%, and the new species *Bean yellow disorder virus* (BnYDV) that broke out in 2004 with occurrence values higher than 34.3% that year. From 2000–2004 an important decrease in TYLCV was observed, along with a slight increase in SBMV and a consistently low occurrence of TSWV. The results of the random surveys confirmed the increased occurrence of virus detected during the directed surveys, and furthermore demonstrated the percentage of incidence for each virus.

Keywords *Phaseolus vulgaris* · Virus incidence · Greenhouse

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Introduction

The main region for producing fresh beans in Europe is located in the provinces of Almería and Granada in south-eastern Spain, using 6,000 ha of plastic greenhouses (occasionally mesh-protected crops) to produce more than 100,000 Tm of fresh beans every year, approximately 50% of the national production. These yields are obtained because of the temperate and sunny climate in this region, and also because of the use of protective greenhouses. The worst problems for intensive protected crops are pests and diseases. Bean is susceptible to more than 270 viruses and at least 35 of them are reported to infect bean naturally (Brunt et al. 1996). Symptoms induced by these viruses are variable as they are influenced by environmental conditions, the particular strain of the virus, mixed infections with other viruses, genetic diversity of bean cultivars, and agronomic practices. Symptoms usually consist of stunting, leaf deformation, mosaic and yellowing (Hall 1994).

Prior to our study, a preliminary study in Spanish bean fields identified several aphid transmitted viruses: *Bean yellow mosaic virus* (BYMV), *Cucumber mosaic virus* (CMV), *Bean common mosaic virus* (BCMV) and bean common mosaic necrotic virus (BCMNV; Saiz et al. 1995). The first two were considered anecdotic; the latter two were considered to be the predominant viruses in Spain (Saiz et al. 1995) and thus were considered common. In addition, two green bean viruses were reported in south-eastern Spain: *Tomato spotted wilt virus* (TSWV), detected by ELISA without any report on the plant symptoms (Lacasa et al. 1991), and TYLCV (Navas-Castillo et al. 1999), which produced a new disease called bean crumble leaf disease that caused severe economic losses in the green bean crops in 1998–1999. This disease is also caused by *Tomato yellow leaf curl Malaga virus* (TYLCMalV), a new species that appeared by natural recombination of TYLCV with the bean non-infecting begomovirus *Tomato yellow leaf curl Sardinia virus* (TYLCSV; Sanchez-Campos et al. 1999; Monci et al. 2002, 2005). Other bean-infecting viruses were identified during the course of our research, such as *Southern bean mosaic virus* (SBMV, Verhoeven et al. 2003) and the new proposed species *Bean yellow disorder virus* (BnYDV, Segundo et al. 2004a). The following study was conducted in south-eastern Spain from 2000–2005, in order to determine the presence and incidence of viruses in bean crops.

Material

Surveys and sample collection

Surveys were conducted in French bean crops at major growing locations in south-eastern Spain. All samples were kept at 4°C and were either mechanically inoculated as soon as possible to test plants, or tested by either direct antibody-sandwich enzyme-linked immunosorbent assay (ELISA) or reverse-transcription polymerase chain reaction (RT-PCR) in 2–5 days, while stock material was stored at −80°C to make dsRNA extraction in 1 month.

Surveys directed to collect symptomatic plants

From 2002 to 2004, leaves from one to three plants displaying similar symptoms (such as mosaic, ring spots, leaf deformation, leaf puckering, spot and/or stunting) were collected at different stages of development from 422 greenhouses (412 plastic greenhouses and 10 mesh-protected structures) in 19 locations (Table 1). Sampled bean plants belonged to 39 different commercial varieties, Donna (Nunhems), Emerite (Vilmorin), Festival RZ (Rijk Zwaan), Helda (several seed companies), Maite RZ (Rijk Zwaan), Nuria (Nunhems), Oriente (Ramiro Arnedo SA) and 32 other varieties from different seed companies (one to six samples per variety), Donna being the predominant variety during the years of the surveys with a mean dominance of 49.5% (Table 2). Our directed surveys included material collected while we were guided by agronomists or growers who were asked to show us plants or crops with virus-like symptoms. We also included all the bean samples sent to the Plant Protection Service Laboratory in Almería. Bean samples were tested by mechanical inoculation with a range of indicator plants, by dsRNA analysis and by geminivirus detection with PCR. Samples with characteristic disease symptoms were analysed for CMV, BCMV, potyviruses, and/or TYLCV. Some negative samples obtained by those tests were analysed for carlaviruses, cucumoviruses and luteoviruses. Positive results allowed us to use new primers to identify SBMV, TSWV, BnYDV and

Table 1 Characteristics of directed surveys to collect symptomatic plants in 2000–2004: locations, number of crops, hectares (ha) and virus detected

Location	No. of crops	Hectares	Number of crops with the indicated virus				w/s
			TYLCV	TSWV	SBMV	BnYDV	
Adra (AL)	40	20	7	2	1	1	29
Albuñol (GR)	15	6.3	4	2	0	0	9
Almería (AL)	6	1.9	0	0	0	0	6
Balanegra (AL)	39	24.4	4	1	5	2	27
Balerma (AL)	67	36.2	15	5	18	4	25
Berchules (GR)	2	0.3	0	0	0	0	2
Berja (AL)	16	6.8	2	1	0	3	10
Canjayar (AL)	2	0.4	1	0	0	0	1
Castell de Ferro (GR)	15	3	0	0	3	1	11
Dalías (AL)	15	6.4	3	0	0	0	12
El Ejido (AL)	95	55.5	26	5	6	8	50
Granada (GR)	14	7.9	1	0	0	0	13
La Mojonera (AL)	18	9.8	5	0	1	1	11
La Rábita (GR)	2	0.5	0	0	0	1	1
Motril (GR)	17	7.7	4	0	0	0	13
Níjar (AL)	15	9	3	1	0	0	11
Pechina (AL)	2	1.5	0	0	1	1	0
Roquetas de Mar (AL)	18	9.7	1	0	2	0	15
Vícar (AL)	24	9.6	10	0	1	2	11
Total	422	216.8	86(20.4%)	17(4.0%)	38(9.0%)	24(5.7%)	257(60.9%)

AL Almería, *GR* Granada, *w/s* number of crops without symptoms

PVuV. At least one isolate of each identified species was sequenced.

Random surveys

In addition to the directed surveys, random surveys were made in 21 greenhouses in 2002 constituting nine main bean production localities (Adra, Balanegra, Balerma, Berja, Castell de Ferro, El Ejido, La Mojonera, Roquetas de Mar and Vícar), and in 12 randomly selected greenhouses in 2005 constituting six main production localities (Adra, Albuñol, Balerma, El Ejido, La Mojonera and Motril). The purpose of the random surveys was to study the incidence of phytopathogenic viruses found in bean crops during the directed surveys of plants showing

Table 2 Relation between bean varieties, the number of crops surveyed in 2000–2004, and the number of crops infected with each virus

Variety	Number of crops	TYLCV	TSWV	SBMV	BnYDV	Total
Donna[a]	209	47	7	31	14	99
Emerite[a]	13	1	2	0	1	4
Festival RZ[a]	32	3	2	0	0	5
Helda[b]	25	8	1	0	0	9
Maite RZ[a]	36	10	0	4	1	15
Nuria	12	1	3	1	0	5
Oriente	23	3	0	2	3	8
32 other varieties	72	12	2	1	5	20
Total	422	85	17	39	24	165

[a] Resistant to *Bean common mosaic virus* according to the producer

[b] Resistant to *Bean common mosaic virus* and *Bean yellow mosaic virus* according to the producers

disease symptoms. Symptoms were recorded from approximately 25% of plants regularly distributed in each crop in 2002 (20,037 plants) and from 10% of plants in 2005 (3,071 plants). In 2002 random samples from approximately 25 plants per greenhouse (total 548 plants) were collected to analyse the presence of known viruses, and in 2005, 116 samples were collected at a rate of 10 plant samples per greenhouse (see Table 5). The symptoms of each collected plant were recorded. All leaves were tested individually by RT-PCR or ELISA.

dsRNA extraction and analysis

Virus-associated double-stranded ribonucleic acid (dsRNA) was extracted from 7 g of symptomatic bean samples or from healthy control samples grown in a growth chamber, and purified by non-ionic cellulose (CF-11, Whatman) chromatography in the presence of 16% ethanol (Valverde et al. 1990). The dsRNA molecular weight was determined by gel electrophoresis in 0.8% agarose. Control dsRNA from a healthy control plant was included in all extractions. Control plants belonged to the following commercial varieties: Donna, Pinto, Black Velvet, Fasili RZ, Helda, Semicorta, Semilarga, Strike, Valenciana and Xera.

Bioassays

Bioassays were made from symptomatic leaves collected in directed surveys to detect and characterise known and new viruses. All the test plants used for the bioassays were grown in pots inside an insect-proofed greenhouse maintained at 15–28°C. Test plants included *Nicotiana benthamiana*, *Nicotiana tabacum* cv. Samsum, *N. tabacum* cv. Xanthi, *Vigna unguiculata*, *Chenopodium quinoa*, *C. amaranticolor*, *Glycine max*, *Vicia faba*, *Phaseolus vulgaris* cv. Donna, *P. vulgaris* cv. Pinto, *Gomphrena globosa*, *Cucumis sativus*, *Datura stramonium*, *Nicotinana clevelandii*, *Nicotiana glutinosa*, and *Pisum sativum*. Sap inoculation of the plants was done by grinding the leaves in 0.03 mM phosphate buffer, pH 8.0 (1:2 *w*/*v*). Extracted sap was rubbed onto leaves pre-dusted with carborundum and then washed with tap water. Plants were inspected for virus symptoms periodically during 4–6 weeks. When bioassays were made to characterise a particular known virus (Table 3), plants without symptoms were analysed by ELISA or RT-PCR. When necessary, re-isolation from inoculated plants was done by inoculating test plants including bean in order to test the infectivity of the virus and to identify latent infections.

Table 3 Host range obtained by mechanical inoculation

Host plant	TSWV	SBMV	TYLCV	BnYDV
Nicotiana benthamiana	0/M (+)	0/0 (−)	0/0 (−)	0/0 (−)
Nicotiana tabacum bv. Samsum	lln/AN (+)	0/0 (−)	0/0 (−)	0/0 (−)
Nicotiana tabacum bv. Xanthi	lln/AN (+)	0/0 (−)	0/0 (−)	0/0 (−)
Vigna unguiculata	llr/MD	0/0 (−)	0/0 (−)	0/0 (−)
Chenopodium quinoa	lln/0 (−)	0/0 (−)	0/0 (−)	0/0 (−)
Chenopodium amaranticolor	lln/0 (−)	0/0 (−)	0/0 (−)	0/0 (−)
Glycine max	lln/N (+)	0/0 (−)	0/0 (−)	0/0 (−)
Vicia faba	lln/N (+)	0/0 (−)	0/0 (−)	0/0 (−)
Phaseolus vulgaris bv. Donna	llr/0(+)	0/M (+)	0/0 (−)	0/0 (−)
Phaseolus vulgaris bv. Pinto	llr/0(+)	lln/0 (−)	0/0 (−)	0/0 (−)
Gomphrena globosa	llr/0 (−)	0/0 (−)	0/0 (−)	0/0 (−)
Cucumis sativus	lln/0 (−)	0/0 (−)	0/0 (−)	0/0 (−)
Datura stramonium	lln/Cl (+)	0/0 (−)	0/0 (−)	0/0 (−)
Nicotinana clevelandii	lln/N (+)	0/0 (−)	0/0 (−)	0/0 (−)
Nicotiana glutinosa	lln/N (+)	0/0 (−)	0/0 (−)	0/0 (−)
Pisum sativum	lln/ N (+)	0/0 (−)	0/0 (−)	0/0 (−)

Local symptoms/systemic symptoms (ELISA or RT-PCR) analysis for non-inoculated leaves, + or −

0 without symptoms, *lln* necrotic local lesions, *llr* reddish local lesions, *Cl* chlorosis, *D* deformation, *M* mosaic, *N* necrosis, *Y* yellowing

Virus detection by ELISA

Serological analyses were carried out following the manufacturer's instructions: DAS–ELISA for BCMV (Loewe), CMV (ADGIA), SBMV (DSMZ), TSWV (Loewe), cucumoviruses (DSMZ); triple antibody-sandwich (TAS–ELISA) for TYLCV (DSMZ); plate-trapped antigen ELISA for potyviruses (AGDIA.) Each plate included a healthy bean tissue sample and an extraction buffer as negative controls. Absorbance values were read at 405 nm using a microplate reader. Tissue known to be positive for a particular virus was used as the positive control. Samples were considered positive when the mean absorbance of duplicate wells exceeded twice the mean absorbance of the corresponding healthy controls.

RT-PCR and sequencing

Nucleic acids for PCR or RT-PCR tests were homogenised from leaf material in Tris–EDTA (pH 8.0), extracted with chloroform-phenol (1:1) and the nucleic acids precipitated with ethanol following standard procedures (Sambrook et al. 1989). Primers used in this work are described in Table 4. RT-PCR amplifications of cucumovirus, potyviruses, carlaviruses, luteovirures, SBMV and BnYDV, as well as PCR amplifications of TYLCV and geminiviruses, were conducted as previously described (Robertson et al. 1991; Badge et al. 1996; Wyatt and Brown 1996; Gibbs and Mackenzie 1997; Choi et al. 1999; Accotto et al. 2000; Verhoeven et al. 2003; Segundo et al. 2004a). Primers for detecting TSWV and PVuV were designed based on sequences retrieved from GenBank. RT-PCR was performed for TSWV and PVuV following standard procedures: First-strand cDNA was synthesised from purified RNA in 20 µl reaction mixes using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. PCR amplifications were performed using direct and reverse primers (100 ng each), and Taq polymerase (Roche) under the following conditions: 94°C, 2 min (one cycle); 94°C, 20 s, 55°C 30 s, 72°C 1 min (35 cycles) and a final step of 10 min at 72°C. For direct sequencing, PCR products were purified using a Wizard PCR purification Kit (Promega, Madison, WI, USA). PCR fragments were sequenced in both strands using an ABI Prism 377 DNA Sequencer. The BLAST programme (Altschul et al. 1997) was used to search for homologies between the obtained sequences and those in the GenBank. Sequences with highest homologies were

Table 4 Oligonucleotides used for identification

Virus	Primers	Sequence	Size (nt)	Reference
TSWV	TSWV777	TGGTGTCATACTTCTTTGGGTC	477	This work
	TSWVA1253	ATCTGGTAGCATTCAACTTCAA		
TYLCV	TY1(+)	GCCCATGTA(T/C)CG(A/G)AAGCC	580	Accotto et al. (2000)
	TY2(−)	GG(A/G)TTAGA(A/G)GCATG(A/C)GTAC		
Carlavirus	CUNI	GGAGTAAC(C/T)GAGGTGATACC	120	Badge et al. (1996)
	CNDA	T21(A/G/C)		
SBMV	SBMV1	TACKCCAAGCAGGAAAGT	870	Verhoeven et al. (2003)
	SBMV2	AATRAGCTCAGCCATAAG		
Potyvirus	POT1	ACCACAGGATCCGGBAAYAAYAGYGGDCARCC	≈1.800	Gibbs and Mackenzie (1997)
	POT2	CACGGATCCCGGG(T)17V		
PV endornavirus	PV3Up	GAATAATGGCATGTGAAGAC	980	This Work
	PV4D	CAAAACCTGCTGGACCTA		
Geminivirus	AC324	GCC(C/T)AT(G/A)TA(T/C)AG(A/G)AAGCC(A/C)AG	550	Wyatt and Brown (1996)
	AC889	GG(A/G)TT(A/G/T)GA(G/A)GCATG(T/A/C)GTACATG		
BnYDV	BnYDVu	TTATGTATGATCTAGGCGGAGGTC	465	Segundo et al. (2004a)
	BnYDVd	CTGGGTCAATGATACAAGTTAGTC		
Cucumovirus	CPTAL3	GACTGACCATTTTAGCCG	950	Choi et al. (1999)
	CPTAL5	(T/C)A(G/C)(T/C)TTT(GAT)(A/G)GGTTCAATTCC		
Luteovirus	Lu1	CCAGTGGTTRTGGTC	530	Robertson et al. (1991)
	Lu4	GTCTACCTATTTGG		

retrieved and used for multiple-sequence alignments with Clustal W (Thompson et al. 1994).

Results

Directed surveys

Presence of viruses, genetic and biological characterization

In 2000–2004, directed surveys were conducted to find plants showing virus symptoms. All collected samples and healthy plants grown in the growth chamber showed a band size of approximately 18 Kb dsRNA (Fig. 1), including Pinto, a commercial bean variety previously described as lacking this band (Wakarchuk and Hamilton 1990). In addition, RT-PCR analysis from purified dsRNA or total nucleic acid extracts using PVuV primers allowed the detection of the cryptic virus *Phaseolus vulgaris endornavirus* PVuV (genus *Endornavirus*, family *Endoviridae*; Fukuhara et al. 2006; Gibbs et al. 2004). A cDNA fragment obtained with PVuV specific primers was sequenced and submitted to the EMBL GenBank as accession AM284175. Analysis showed 100% identity to the existing PVuV sequence X16637 and 98.6% identity with the equivalent sequence of AB185245.

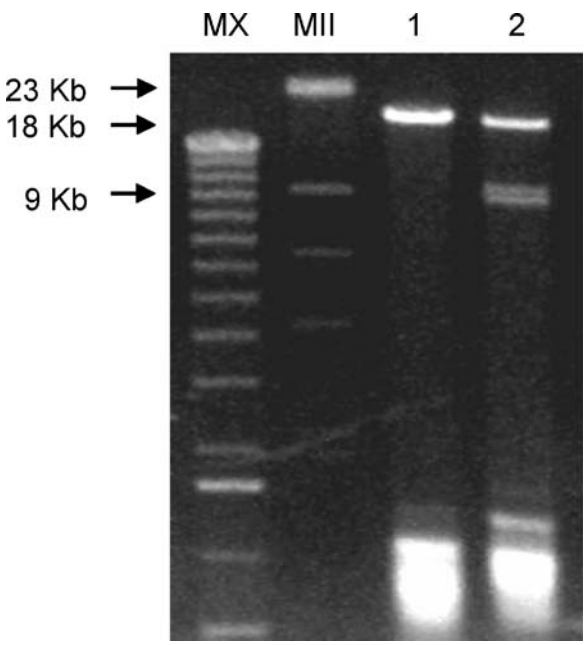

Fig. 1 PVuV and BnYDV dsRNA bands. *MX* and *MII* molecular weight markers, *line 1* healthy bean plant, displaying a ~18 Kb PVuV dsRNA band, *line 2* bean plant infected with BnYDV, displaying the large PVuV band and two smaller BnYDV bands

In these surveys we found that only 39.1% of the sampled greenhouses showed phytopathogenic viruses. Those viruses were: TYLCV (genus *Begomovirus*, family *Geminiviridae*) with an infection rate of 20.4%; SBMV (genus *Sobemovirus*, family *Sobemoviridae*) with an infection rate of 9.0%; TSWV (genus *Tospovirus*, family *Bunyaviridae*) with an infection rate of 4.0%; BnYDV (a tentative species of genus *Crinivirus*, family *Closteroviridae*) with an infection rate of 5.7%. BnYDV was discovered in 2004, and our surveys showed infection values of 34.3% that year. But since our analyses only began in the latter part of the year, the actual infection rate was probably higher. SBMV and BnYDV were associated with typical dsRNA patterns: a 4.1 kb band belonging to SBMV (Verhoeven et al. 2003) and two ~9 Kb bands corresponding to BnYDV (Fig. 1). Virus identities were confirmed by sequence comparison with GenBank accessions (data not shown). TYLCMalV presence was not differentiated from TYLCV positive samples. Plants that displayed virus-like symptoms but which tested negative by various methods (approximately 60.9% of the crops) were considered free of phytophathogenic viruses (Fig. 2). Identified viruses were biologically characterised by mechanical inoculation of a range of indicator plants (Table 3). TSWV mechanically inoculated on bean did not reproduce symptoms even though infective virus was found by means of re-isolation and inoculation in *Nicotiana tabacum* bv. Samsum and *N. tabacum* bv. Xanthi, where it produced typical TSWV symptoms and was RT-PCR positive.

Symptoms

The symptoms identified in bean plants naturally infected by TYLCV were leaves curling inward (Fig. 3a) or outward (Fig. 3b), and pod deformation (Navas-Castillo et al. 1999). Young plants infected by TYLCV showed a shortening of internodes and dwarfing. TSWV-infected plants showed the following symptoms after high thrips infestation: leaf deformation, occasional half-lateral leaf deformation (Fig. 3c and d), ring spots (Fig. 3e), yellowing in

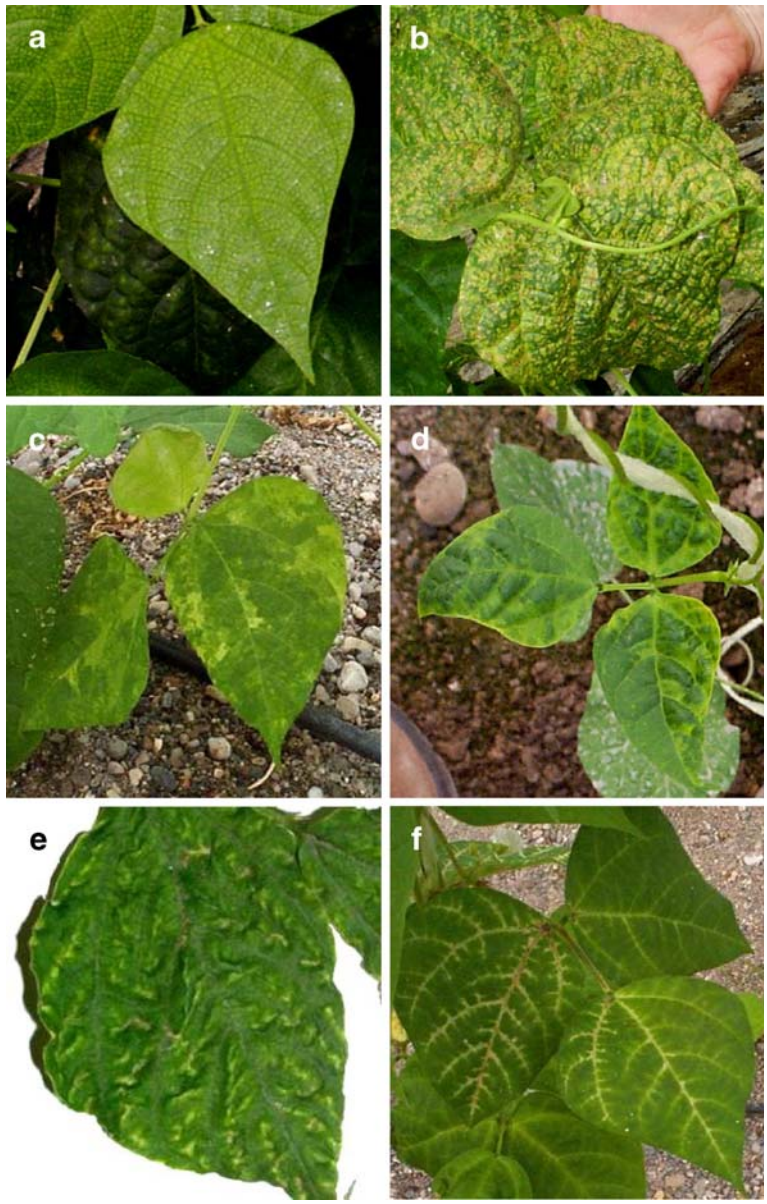

Fig. 2 **a**–**f** Plants displaying symptoms not associated with viruses

leaves and reddish nerves in back leaves (Fig. 3c), stem necrosis. It was also observed that TSWV-infected bean plants produced new stems from branching and achieved a good final yield, while BnYDV-diseased plants displayed leaf yellowing that progressed to necrosis and fruit deformation leading to a poor final yield (Fig. 3f–h). Symptoms induced by BnYDV are easily mistaken for other physiological alterations caused by aging or environmental changes, thus making symptomological detection more difficult. Finally, symptoms caused by SBMV consisted of smooth mosaic on the leaves followed by deformations of variable intensity, plus pod deformation (Verhoeven et al. 2003; Segundo et al. 2004b; Fig. 3i).

Variations in viral prevalence

Directed surveys in 2000–2004 (Fig. 4) recorded changes in the prevalence of some viruses. SBMV incidence increased from 1.2% of infected greenhouses in 2002 to 21.4% in 2004. TYLCV incidence

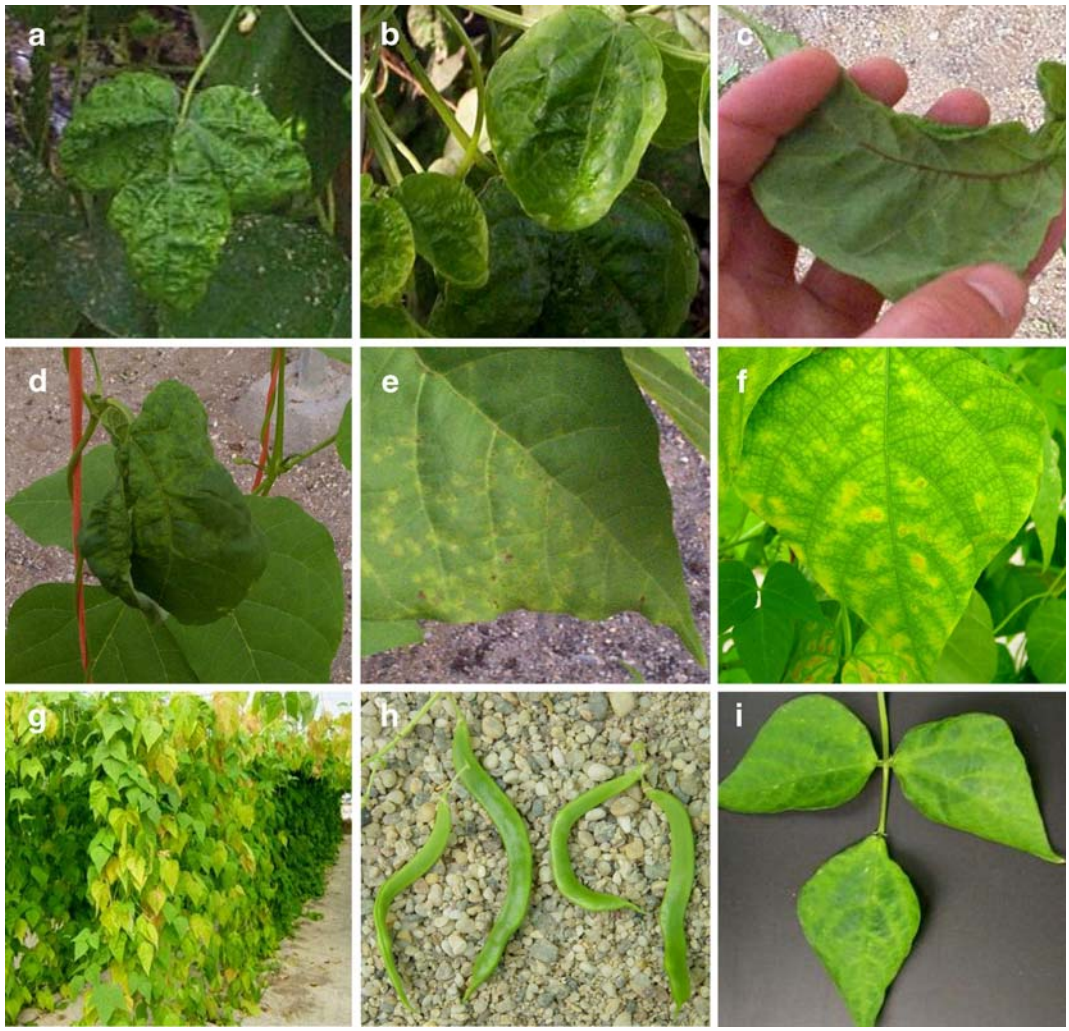

Fig. 3 Plants displaying symptoms that are characteristic of viruses found in French beans in southern Spain. **a** and **b** TYLCV, **c**–**e** TSWV, **f**–**h** BnYDV, **i** SBMV

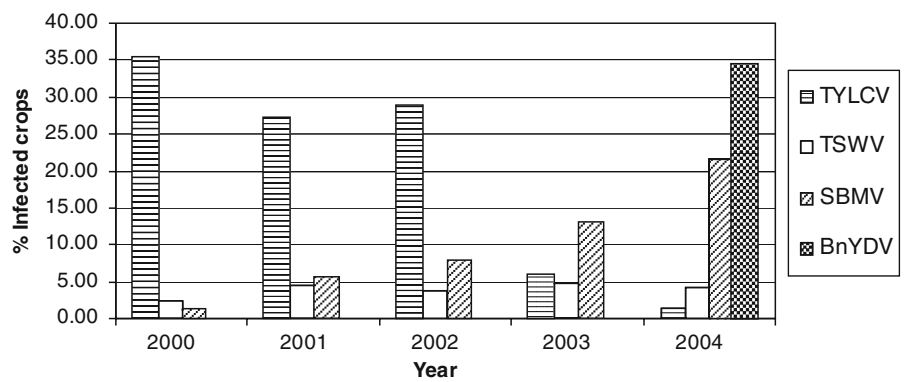

Fig. 4 Variation in levels of viruses found in directed sampling to collect plants with symptoms attributable to bean virus, 2000–2004

Table 5 Virus incidence in sampled plants from random surveys

Pathogenic viruses	2002 (21 crops)		2005 (12 crops)	
	Inspected plants, *S*(%)	Analysed plants, *S*(%)/*A*(%)	Inspected plants, S(%)	Analysed plants, *S*(%)/*A*(%)
TYLCV	199 (1)	20(3.6)/24(4.4)	5(0.2)	1(0.9)/1(0.9)
SBMV	209(1)	7(1.3)/16(2.9)	0(0)	0(0)/0(0)
TSWV	166(0.8)	14(2.6)/34(6.2)	167(5.4)	4(3.4)/10(8.6)
BnYDV	0(0)	0(0)/0(0)	520(17.0)	47(40.5)/55(47.4)
TSWV + BnYDV	0(0)	0(0)/0(0)	19(0.6)	5(4.3)/5(4.3)
Negatives	19463(97.1)	507(92.5)/474(86.5)	2,360(76.9)	59(50.9)/45(38.8)
Total	20,037	548	3,071	116

All inspected and analysed plants are grouped by the year of random surveys. Twenty-five percent of plants regularly distributed within a crop in 2002 and 10% in 2005 were visually inspected each year. Among them about 25 plants/crop were analysed in 2002 and about 10 plants/crop in 2005.

S(%) observed plants displaying virus symptoms (percentage), *A(%)* analysed plants giving positive results (percentage)

dropped from a 35.4% infection rate in greenhouses in 2002, to a rate of 1.4% in 2004, while the sudden presence (34.3% or more, as noted above) of BnYDV was observed in 2004. TSWV incidence was stable (around 5%) throughout the period surveyed. SBMV was more prevalent in areas with greater concentrations of crops (Balanegra and Balerma). In the 19 surveyed localities we were unable to determine a link between bean variety and viral prevalence over time.

Random surveys

The presence or absence of symptoms was recorded (Table 5) by our random survey of 20,037 plants in 21 greenhouses in 2002 and 3,071 plants in 12 greenhouses in 2005. The results of testing show that TSWV, SBMV and TYLCV were all present in about 1% of plants in 2002. But in 2005 the new virus BnYDV appeared in 17.6% of all observed plants, TSWV increased to 5.4%, and the percentage of TYLCV-diseased plants decreased notably from 1% to 0.2%. The appearance of infections caused by a mixture of BnYDV with TSWV was also noted in our survey.

In both 2002 and 2005 we observed a high degree of latent infection in those crops from the random surveys whose visual infection rate was >25%. In 2002, for example, of the plants in the crop which

Table 6 Number of infected crops and percentage of incidence of viruses in each crop in random surveys

Pathogenic viruses	2002 (21 crops)					2005 (12 crops)				
	Numbers of crops with symptoms and percentage of infection				Total crops infected	Numbers of crops with symptoms and percentage of infection				Total crops infected
	0≤1%	1–9%	10–25%	≥26%		≤1%	1–9%	10–25%	≥26%	
TYLCV	5[a]	0	0	1	6	0	0	0	0	0
SBMV	0	0	0	1	1	0	0	0	0	0
TSWV	1	0	0	1	2	0	0	0	1	1
BnYDV	0	0	0	0	0	0	3	2	3	8
TYLCV + BnYDV	0	0	0	0	0	0	1[b]	0	0	1
TSWV + BnYDV	0	0	0	0	0	0	1	0	1	2
Total	6	0	0	3	9	0	5	2	5	12

[a] Number of greenhouses with one or two viruses (not necessarily in the same plant)

[b] Sampled crop showing five plants with TYLCV symptoms and a higher number of plants with BnYDV symptoms, without plants double-infected.

showed a high rate of infection by SBMV, 64% (16 out of 25 plants) tested positive for SBMV while only 28% (seven out of 25 plants) showed observable symptoms (Table 5). In 2005 there was a crop from which 83% of the samples (10 out of 12 plants) tested positive for TSWV while only 33% (four out of 12 plants) showed symptoms (Table 5).

The overall presence of virotic crops increased, from 42.9% (nine out of 21) of the greenhouses surveyed in 2002 to 100% (all 12) of the greenhouses surveyed in 2005 (Table 6). The decline in TYLCV prevalence described above in directed surveys was also observed in random surveys (Table 6) because six out of 21 greenhouses (28.6%) in 2002 tested positive for infection, whilst in 2005 only one out of 12 (8.3%) tested positive for TYLCV. TSWV was detected in crops from two greenhouses (9.5%) in 2002 and from three greenhouses (25%) in 2005. SBMV was not detected in random samplings, although the virus was found in directed surveys that focused on collecting diseased plants during 2005 (data not shown).

With respect to the incidence of virus within those crops selected at random (Table 6), in 2002 only 14.3% (three out of 21) showed a high incidence (≥10%): one with TSWV, another with SBMV and a third with TYLCV. By contrast, 58.3% of the crops (seven out of 12) in 2005 showed high incidences: one crop with TSWV and six with the new virus BnYDV. One of these crops showed a small percentage of plants with mixed infections of both BnYDV and TSWV.

Discussion

Protected green bean greenhouses in the south-eastern Spanish provinces of Almería and Granada have a great economic impact on the Spanish agricultural economy. Nevertheless, up to the time of our study, no systematic studies had been carried out to identify bean viruses and their incidence in this crop. Consequently, in some cases unknown diseases have been thought to be caused by viruses.

During a 6-year survey period, a cryptic virus and four phythopathogenic viruses have been identified. PVuV cryptic virus is a recently described species from the *Endornavirus* genus (Gibbs et al. 2004). Endornaviruses have been described in plants, chromista and fungi (Fukuhara et al. 2006; Hacker et al. 2005; Osaki et al. 2006) and phylogenetic analyses have indicated that these viruses share a common ancestor with an alpha-like supergroup of RNA viruses (Gibbs et al. 2000). Endornaviruses show large endogenous RNAs (>10 kb) at a low constant concentration not associated with virus-like particles. They are transmitted via seed and pollen, although horizontal transmission has been identified in a fungus (Ikeda et al. 2003). Because the completely sequenced dsRNAs show only one long open reading frame with conserved motifs for RNA-dependent RNA polymerase and RNA helicase, the dsRNAs are compared to large plasmid-like replicons. None of these dsRNAs have an obvious effect on the phenotype of the host, with the exception of the cytoplasmic male sterility of *Vicia faba* and the hypovirulency of a strain of *Helicobasidium mompa* (Osaki et al. 2006). Furthermore no phenotypic effect has been associated with *Phaseolus vulgaris* dsRNAs.

In our study, *Phaseolus vulgaris* showed a ~18 Kb dsRNA band as previously reported by Wakarchuk and Hamilton (1990). We sequenced a dsRNA fragment of 941 nt and determined that it was 99.5% identical to the equivalent region of the two adjacent fragments described in Genbank (accession no. X16637 and AB185245), one of which had already been described as belonging to PVuV. Although some previous work indicates that the ~18 kb band does not appear in all beans (Wakarchuk and Hamilton 1990), its appearance in all of our specimens and varieties, coupled with our RT-PCR results, suggests otherwise. Thus our data contribute to a better knowledge and awareness of the distribution of PVuV in bean crops throughout the world.

Another important observation is that of all the greenhouses with plants that showed virus-like symptoms, only 39.1% were infected by pathogenic viruses, while the remaining 60.9% showed no such infection after serological, molecular and biological tests of their plants. Symptomatic plants in which no pathogenic viruses were detected by any of the methods came from crops where there were very few such plants (one to five plants per crop), or where they appeared in only a small area of the crop, or in some cases where all the plants were symptomatic. The case of few symptomatic plants suggests factors such as individual seeds with genetic disorders. Symptoms appearing in only a small area suggest

either agronomic factors such as abuse of herbicides and fertilizers or the presence of other biological agents. The same agronomic factors could also be the cause of symptoms in entire crops, but they could as well be caused by environmental factors like abrupt changes in temperature and humidity, to which beans are very sensitive.

TSWV and TYLCV were identified in Spain before the start of the current study (Lacasa et al. 1991; Navas-Castillo et al. 1999), whereas SBMV was first identified in Spain during the course of this research (Verhoeven et al. 2003). BnYDV was found as a new tentative species in south-eastern Spanish bean greenhouses (Segundo et al. 2004a). Up to now, BCMV and BCMNV have been considered the predominant viruses in bean crops in Spain (Saiz et al. 1995). Our data show that BCMV and BCMNV are uncommon in plastic greenhouses, probably because recent improvements in physical and chemical controls can eliminate the aphids that transmit those viruses, and commercial seeds are now certified as virus-free or virus-resistant. TSWV isolated from bean had biological (Table 3) and genetic characteristics similar to isolates from other vegetable crops in the area (unpublished data). Thrips (*Frankliniella occidentalis*) were usually found in bean greenhouses located near other TSWV-diseased crops such as tomatoes and peppers. In spite of the severe TSWV disease, however, bean plants recovered after the insect population was controlled. New buds from the first branches were without symptoms and the yield remained at a high level. It is noteworthy that although in our study we were able to mechanically transmit TSWV to bean plants (detected by ELISA), they nevertheless displayed no disease symptoms even though the infective virus could always be re-isolated. Furthermore, although *P. vulgaris* has been described as a natural host for TSWV, we have not been able to find in the literature a description of TSWV symptoms in bean (Best 1968; Sether and De-Angelis 1992; Parrella et al. 2003). This absence makes us conclude that symptoms in bean caused by TSWV in south-eastern Spanish greenhouses could be related to other factors: toxins, strains of thrips, plant defence mechanisms, hypersensitivity to a massive attack of thrips, and/or environmental factors.

Our study also provides a better understanding of the evolution of bean diseases in protected horticulture. At the beginning of our research, TYLCV was the main virus disease for French bean in south-eastern Spain. By 2004, our surveys indicated a much lower incidence. This could be due to an increased incidence of TYLCSV which is hosted by tomato crops in the area, to an increase of recombinant begomoviruses (Monci et al. 2002) that cannot infect bean, or to a lack of competitiveness with other viruses such as BnYDV.

Our directed surveys from 2000–2004 indicated a steady increase in SBMV. But contrary to this trend, our random surveys in 2005 did not detect SBMV, even though we had found the virus in one diseased crop during that year of directed surveys (data not shown.) The reason for this could be that after we reported that SBMV was the causal agent of the disease, agronomists and growers took sufficient control actions as soon as infections appeared to prevent contact transmission between plants either within a greenhouse or between greenhouses. Furthermore, growers began to plant different crops like pepper or melon, in order to eliminate bean viruses from the soil. This worked because SBMV is a beetle-, seed- and contact-transmitted virus that persists in soil. Contact transmission has been reported in Spain to be a fast mechanism during harvesting and soil transmission for the next bean crop (Verhoeven et al. 2003). In several areas SBMV appeared in greenhouses where it had not been observed before and where mechanical transmission was not known to have occurred. Beetles from the *Chrysomelidae* family are reported to transmit SBMV (Walters 1969), but this insect has not been identified in the greenhouses of south-eastern Spain. Thus the sudden outbreak of SBMV has been associated with seed contamination that later spread by mechanical transmission (Verhoeven et al. 2003).

Having discussed several specific viruses in directed surveys, we also need make a few observations from our random surveys in 2002 and 2005. First, the data showing the increased overall virus incidence and the increased number of greenhouses with high incidence resulted from the appearance and prevalence of BnYDV in 2005. Second, the disparity between the directed and random survey data for TSWV (no significant variation in directed surveys vs. increased incidence in random surveys) may have been due to a sampling bias or to an increased incidence of the disease in other crops like pepper in 2005 (unpublished data.)

In conclusion, our research provides more current data regarding viruses that cause diseases in green beans cultivated in south-eastern Spain, and presents the evolution of these diseases along with the appearance of several new viruses in Spain. Having identified new viruses, and knowing their mechanisms of transmission and their potential for high rates of infection, our study offers insight into how these viruses can be controlled in order to avoid these diseases and their subsequent role in the reduction in crop yields.

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