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Southern bean mosaic virus the causal agent of a new disease of *Phaseolus vulgaris* beans in Spain

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Abstract

Southern bean mosaic virus (SBMV) has been identified as the cause of a new disease in greenhouse-cultivated common bean (*Phaseolus vulgaris*), in the south-east of Spain. The identification was based on host range comparisons, morphological and serological characteristics of the virus, the size of its dsRNA species and the nucleotide sequence of an 810-bp fragment from ORF2. The virus could be clearly discriminated from the related sobemovirus *Southern cowpea mosaic virus*. This is the first report of SBMV in Spain.

Introduction

Since 1998, symptoms of unknown origin have been observed in greenhouse-cultivated common bean (*Phaseolus vulgaris*) in the south-east of Spain. The severity of the symptoms varied between different bean varieties. The pods showed mosaic patterns followed by distortion (Figure 1a), and the number and size of the seeds were reduced. The leaves were slightly distorted, showing mild chlorosis and marked veins (Figure 1b). The first symptoms in young plants often appeared simultaneously at several locations in the greenhouse. In course of time, neighbouring plants also became diseased, especially after harvesting the first pods. During preliminary surveys covering over one hundred greenhouses, diseased bean plants were found in approximately 10% of the greenhouses with infections rates ranging from 1% to 99% of the plants. The present paper describes the identification of *Southern bean mosaic virus* (SBMV) as the cause of the observed symptoms in common bean, based on host range, particle morphology, serology, the size of its dsRNA species and the nucleotide sequence of an 810-bp fragment from ORF2.

SBMV is a sobemovirus that causes mosaic and mottle diseases in common bean (Tremaine and Hamilton, 1983). The virus is characterised by isometric particles (30 nm), which encapsidate a single-stranded positive-sense genomic RNA of approximately 4100 nucleotides. It was first reported in common bean in Louisiana and California (USA) in 1943 (Zaumeyer and Harter, 1943) and has since been detected in South America and France (Tremaine and Hamilton, 1983). Until recently, SBMV was considered to consist of two strains that differ with regard to their host range: the bean (type) strain (SBMV-B) that systemically infects many varieties of common bean but not cowpea (*Vigna unguiculata*), and the cowpea strain (SBMV-C) that systemically infects cowpea but not bean (Shepherd and Fulton, 1962). As these strains also differ serologically and their overall sequence homology appears low (55%), they have now been designated as different

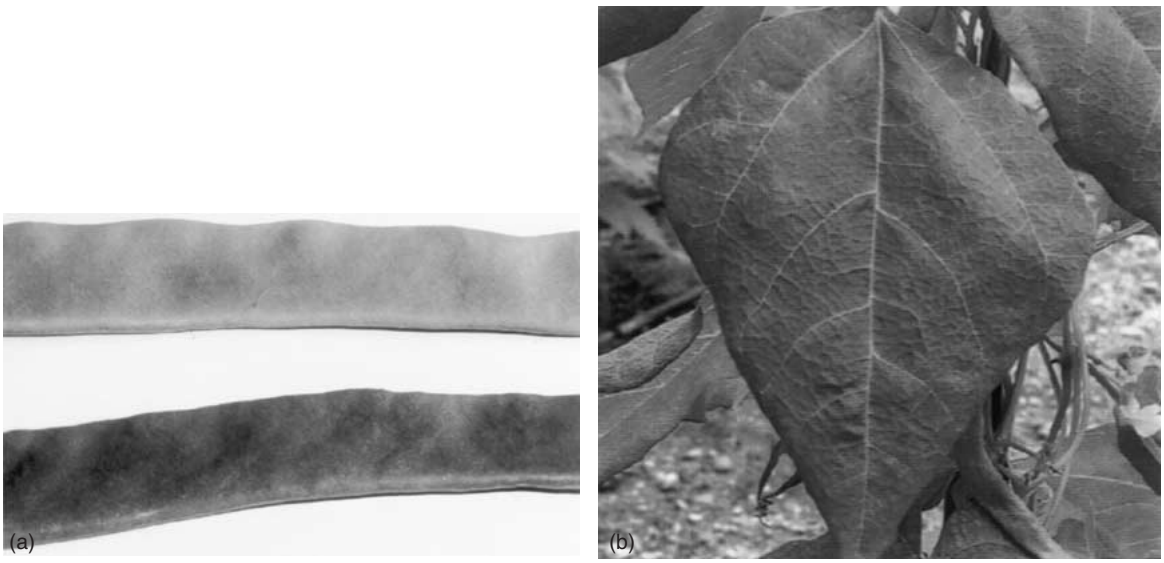

Figure 1. Symptoms caused by SBMV in greenhouse grown *P. vulgaris* 'Donna'. (a) Healthy pod (upper) and infected pod (lower) and (b) Infected leaf displaying marked veins.

species i.e., SBMV and *Southern cowpea mosaic virus* (SCPMV) (Tamm and Truve, 2000).

Materials and methods

Virus isolates. Virus isolates were obtained from different varieties of common bean grown in greenhouses in the provinces of Almeria and Granada. For reference and comparison the *P. vulgaris* isolate PV-0100 of SBMV originating in Colombia was kindly provided by S. Winter (German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany). All isolates were propagated by mechanical inoculation to *P. vulgaris* 'Donna', 'Dubbele Witte Zonder Draad' or 'Saxa'. For comparison an isolate of bean mild mosaic virus (BMMV) was obtained from Plant Research International, Wageningen, the Netherlands.

Comparative study on mechanically inoculated plants. Two virus isolates, PD20007079 and PD21005561, were mechanically inoculated on *Arachis hypogaea*, *Capsicum annuum* 'Westlandse Grote Zoete', *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus* 'Hokus', *Datura stramonium*, *Nicotiana benthamiana*, *N. glutinosa*, *N. hesperis* (accession 67A), *N. occidentalis* (accession P1), *N. tabacum* 'White Burley', *Lycopersicon esculentum* 'Moneymaker', *P. vulgaris* 'Dubbele Witte Zonder Draad' and 'Pinto', *Pisum sativum* 'Kelvedon Wonder', *Vicia faba* 'Witkiem' and *V. unguiculata* 'Black Eye' and 'Early Red' (Verhoeven and Roenhorst, 2000). Symptomless plants of legumes were indexed for latent infections by mechanical inoculations to *P. vulgaris* 'Pinto'.

Preparation of single lesion isolates for inoculation on P. vulgaris 'Donna'. Individual local lesions of *P. vulgaris* 'Pinto' infected with the isolate PD21005561 were mechanically inoculated on healthy seedlings of 'Pinto'. Likewise, local lesions of these plants were inoculated individually on *P. vulgaris* 'Dubbele Witte Zonder Draad'. Systemically infected leaves of the latter plants were used for the inoculation of *P. vulgaris* 'Donna'. Plants of 'Donna' were observed for virus symptoms for 8 weeks.

Electron microscopy. Virus particles were visualised after incubating Pioloform-carbon coated copper grids with crude plant extracts for 5 min. The grids were washed with distilled water and negatively stained with 1% aqueous uranyl acetate.

Antisera. Three antisera to bean isolates of SBMV were obtained from DSMZ, Braunschweig, Germany (AS-0033, homologous to PV-0100 from Colombia), H.E. Waterworth, Beltsville, Maryland, USA (=ATCC PVAS 37) and E.W. Kitajima, Brasilia, Brazil

(Cupertino et al., 1982). Antiserum to the cowpea strain of SBMV was obtained from ATCC, Beltsville, Maryland, USA (PVAS 114) and that to BMMV, produced by H.E. Waterworth (#36), from R. Koenig, Braunschweig, Germany.

Immunoelectron microscopy. For immunosorbent electron microscopy (ISEM, Milne and Lesemann, 1984) Pioloform-carbon coated grids were coated for 5 min with antisera diluted 1 : 1000 in 0.1 M phosphate buffer pH 7.0 (PB). Particles were trapped on antiserum-coated grids during 15 min incubation with crude plant extracts of *P. vulgaris* 'Saxa' or 'Dubbele Witte Zonder Draad'. For decoration tests (Milne 1993) adsorbed or antibody-trapped particles were incubated with antisera diluted 1 : 50. For decoration titer determinations, two-fold dilution series of the respective antisera were used in the range from 1 : 50 to 1 : 12,800. The highest dilution step resulting in decoration was recorded as the titer of the respective antiserum–antigen combination.

Double-stranded RNA purification. Total double-stranded RNA (dsRNA) from healthy and SBMV-infected plants was purified from total nucleic acid extracts by non-ionic cellulose (CF-11, Whatman) chromatography in the presence of 16% ethanol (Valverde et al., 1990). The size was determined by electrophoresis in agarose (0.8%).

RT–PCR. Two oligonucleotide primers were designed after multiple alignments of sobemovirus sequences AF055887 (SBMV-B^{ARK}) (Lee and Anderson, 1998), AF055888 (SBMV-S) (Lee and Anderson, 1998), L34672 (SBMV-B) (Othman and Hull, 1995), AY004291 (*Sesbania* mosaic virus, SeMV) (Lockesh et al., 2001) and M23021 (SCPMV) (Wu et al., 1987), using the Clustal W program (Thompson et al., 1994). The primers SB1 5′-TACKCCAAGCAGGAAAGT-3′ and SB2 5′-AATRAGCTCAGCCATAAG-3′ amplified a 870-bp DNA fragment from an internal region close to the 3′ end of the ORF2-coding sequence of the SBMV genome. For RT–PCR leaf material (0.1 g ml^{-1}) was homogenised in Tris–EDTA (pH 8), extracted with chloroform–phenol (1 : 1), and total RNA was precipitated with isopropanol following standard procedures (Sambrook et al., 1989). The RNA was primed with SB2 and used for the first-strand cDNA synthesis with 100 units of Superscript II™ RNase H$^-$ Reverse Transcriptase (Life Technologies, Barcelona, Spain) following the manufacturer's instructions. PCR amplification was performed using SB1 and SB2 (100 ng each), and Taq polymerase (Roche, Barcelona, Spain) under the following conditions: 94 °C, 2 min (1 cycle); 94 °C, 30 s, 50 °C, 1 min, 72 °C, 1 min (35 cycles) and 72 °C, 10 min (1 cycle).

Cloning and sequence analysis. The PCR products were purified (PCR Clean Up Kit, Roche) following the manufacturer's instructions, and then ligated to pGEM® T EASY Vector (Promega, Madison). These plasmids were used to transform competent DH5α cells (Life Technologies) of *Escherichia coli* by heat shocking. Transformed clones were selected according to usual protocols (Sambrook et al., 1989) and cloned cDNA fragments were sequenced on both strands in an ABI Prism 377 DNA Sequencer. BLAST (Altschul et al., 1990) was used to search for homologies between the obtained sequences and those in GenBank. Sequences with highest homologies were retrieved and used for multiple-sequence alignments with Clustal W (Thompson et al., 1994) and subsequent construction of unrooted phylogenetic trees using the neighbour-joining algorithm in Bioedit (Hall, 1999). These were visualised using TreeView (Page, 1996).

Results

Symptomatology of mechanically inoculated plants. Plants of *P. vulgaris* 'Pinto' inoculated with isolates PD20007079 and PD21005561, showed necrotic lesions on the inoculated leaves (Figure 2), but did not become systemically infected. *Phaseolus vulgaris* 'Dubbele Witte Zonder Draad' had no local symptoms but became systemically infected, showing symptoms both in leaves and pods. The varieties 'Donna' and 'Saxa' used for maintaining the virus isolates, reacted in a similar way. Both varieties of *V. unguiculata* 'Black Eye' and 'Early Red', as well as all other test plant species, did not become infected.

Four single isolates of PD22005561 were obtained after mechanical inoculation of single local lesions of *P. vulgaris* 'Pinto' on healthy seedlings of the same plant species. One of these isolates was used for mechanical inoculation of bean 'Donna'. Leaves and pods of all inoculated plants of 'Donna' developed virus symptoms similar to those observed in Spanish greenhouses.

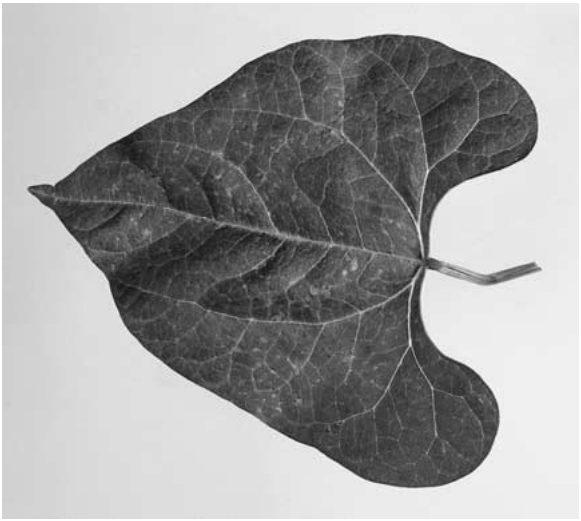

Figure 2. Leaf of *P. vulgaris* 'Pinto' showing small necrotic lesions 4 days after mechanical inoculation with isolate PD20007079.

Particle morphology and immunoelectron microscopy. Crude extracts of infected common bean leaves revealed moderate numbers of isometric particles c. 25–30 nm in diameter with angular outlines and a smooth surface structure. Empty shells were observed in variable amounts. The morphology of the virus particles appeared typical for sobemoviruses. Consequently, two virus isolates from common bean in Spain, PD20007079 and PD21005561, and the reference isolate of SBMV, PV-0100, were tested for their reaction with different antisera to SBMV using immunoelectron-microscopical techniques. All isolates were effectively trapped by the three antisera to bean isolates but also by the antiserum to a cowpea isolate. At antiserum dilutions of 1 : 50 all three isolates showed a medium to strong decoration (antibody attachment to particle surfaces) with all four antisera. This indicates that the virus isolates are related serologically.

Decoration titers were determined in order to quantify the reactions of the four antisera to SBMV with the three virus isolates (Table 1). These titers revealed differences in terms of the absolute reactivities and of the specificities of the viruses and antisera, respectively. AS-0033 and PVAS 114 reached the highest and lowest titers, respectively. Two antisera to bean isolates, i.e., AS-0033 and the one from Waterworth, produced identical titers with all isolates. The antiserum to the Brazilian bean isolate revealed slightly differing

Table 1. Serological differentiation of SBMV isolates by decoration titers using four antisera to SBMV strains

Antisera	Antigens isolated from *P. vulgaris*		
	Spain 20007079	Spain 21005561	Colombia PV-0100 DSMZ
Bean Colombia, DSMZ AS-0033	6400*	6400	6400
Bean, Waterworth (=ATCC PVAS 37)	1600	1600	1600
Bean Brazil, Lin (Kitajima)	1600	3200	1600
Cowpea, ATTC PVAS 114	400	1600	400

*Reciprocal decoration titers.

reactions with the three bean isolates whereas that to the cowpea isolate PVAS 114 from USA produced the lowest and most differentiated titers among the antisera: isolates 20007079 and PV-0100 from bean in Spain and Colombia, respectively, reacted one or two dilution steps weaker than isolate 21005561 from bean in Spain.

In order to prove the exclusive presence of SBMV and to avoid possible misinterpretations caused by a putative unrecognised contamination with BMMV, all three SBMV isolates were tested in immunoelectron microscopy using an antiserum to BMMV. In ISEM and decoration tests no decorated particles were observed. In addition, in ISEM plus decoration tests none of the four antisera to SBMV (raised from bean as well as from cowpea) was found to react with authentical BMMV. However, BMMV particles reacted strongly with their homologous antiserum. Moreover, the granular surface structure of BMMV particles clearly differed from the smooth surface structure of SBMV particles.

DsRNA. RT–PCR and nucleotide sequence analyses. Only samples from infected *P. vulgaris* plants, collected from the field or from mechanically inoculated plants, yielded a dsRNA product of approximately 4.1 kb (Figure 3), which correlates with the predicted size of the genome of SBMV. An additional band of approximately 20 kb was found both in samples from healthy and infected plants.

RT–PCR on total RNA extracts from infected plants, but not from healthy plants, produced amplicons of the expected size (870 bp) (Figure 4). These were sequenced and compared with other isolates of SBMV and related viruses (Table 2). The sequence of the

Table 2. Percentage genome sequence identities (810 bp of ORF2) of SBMV isolates and related viruses

	SBMV-ALM	SBMV-B^{ARK}	SBMV-S	SBMV-B	PV-0100	SeMV	SCPMV
SBMV-ALM		96.4	96.3	93.0	87.8	79.6	66.0
SBMV-B^{ARK}			99.8	94.1	88.1	80.3	65.6
SBMV-S				93.9	88.2	80.1	65.7
SBMV-B					85.6	78.3	64.2
PV-0100						81.1	66.5
SeMV							64.4
SCPMV							

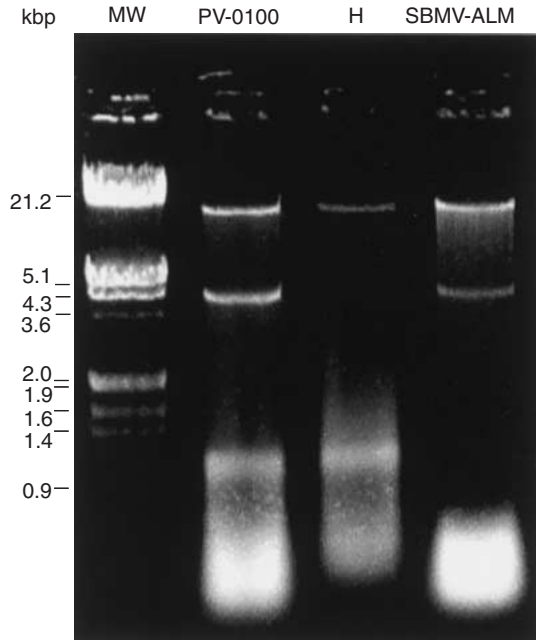

Figure 3. Electrophoresis of double-stranded RNA from healthy plants (H), plants infected with PV-0100 and with the Spanish isolate (SBMV-ALM), respectively. Ethidium-bromide stained 0.8% agarose gel in TBE. MW = DNA molecular weight marker III (Roche).

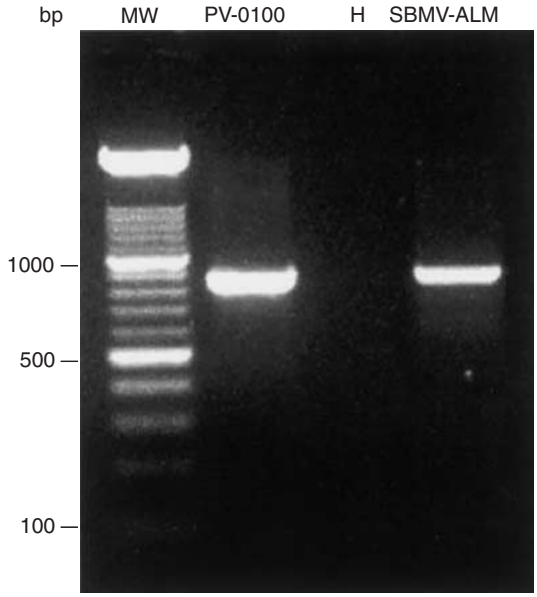

Figure 4. RT–PCR with primers SB1 and SB2 for healthy plants (H), plants infected with PV-0100 and with the Spanish isolate (SBMV-ALM), respectively. Ethidium-bromide stained 2% agarose gel in TBE. MW = DNA molecular weight marker XIV (Roche).

Spanish field isolate (SBMV-ALM, Genbank Accession Number AJ414558) showed the highest homology (96%) with SBMV-B^{ARK} and SBMV-S, followed by SBMV-B (93%). Lower identities were found with PV-0100 (88%), SeMV (80%) and SCPMV (66%). The phylogenetic relationship of SBMV-ALM and five other sobemovirus isolates based on the sequence homology of the corresponding 810 bp nucleotide fragment revealed that SBMV-ALM clusters in the same group as SBMV-B^{ARK}, SBMV-S and SBMV-B, whereas SeMV, PV-0100 and SCPMV form different clusters (Figure 5).

Discussion

SBMV is considered to comprise two separate species, SBMV (type SBMV-B) and SCPMV (Tamm and Truve, 2000). The former systemically infects many *P. vulgaris* varieties but not *V. unguiculata*, whereas the latter systemically infects *V. unguiculata* and *P. sativum* but not *P. vulgaris*. Furthermore, other SBMV isolates have been described (from Ghana or the Ivory Coast, and from Mexico) which infected both common bean and cowpea (Othman and Hull, 1995; Tremaine and Hamilton, 1983).

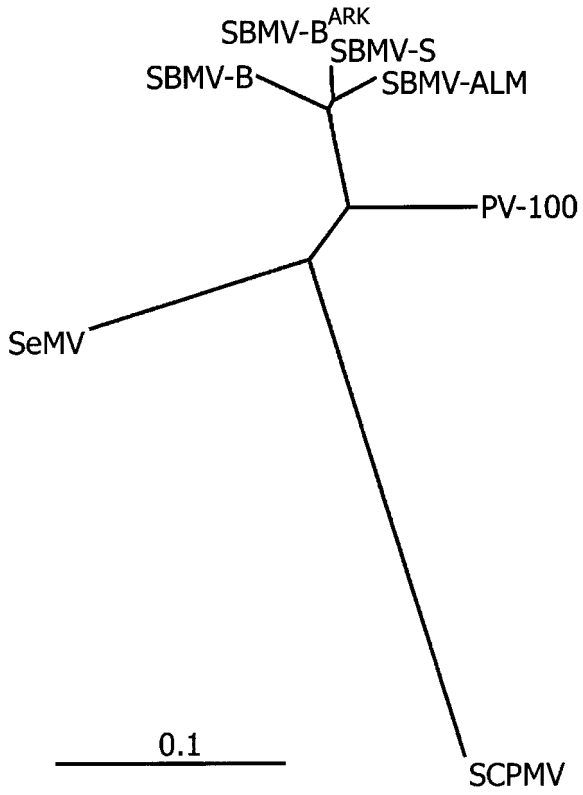

Figure 5. Phylogenetic neighbour-joining tree, showing the relationship between isolates of SBMV (5), SeMV (1) and SCPMV (1), based on 810 bp homologue nucleotide genome sequence from ORF2.

Evaluation of host range, particle morphology and serological characteristics indicated that the virus isolated from infected plants of common bean is identical to SBMV, type SBMV-B (Grogan and Kimble, 1964). Presence of BMMV was unlikely, as this virus systemically infected *P. vulgaris* 'Pinto' but did not evoke local lesions (Waterworth, 1981). With regard to BMMV, also the morphological and serological comparison with different isolates of SBMV did not indicate the presence of this virus. In contrast, the three antisera to SBMV isolates from common bean in Colombia, USA and Brazil, reacted strongly with both common bean isolates from Spain and isolate PV-0100 from Colombia. The weaker reaction of the antiserum to the cowpea strain of SBMV with one of the two Spanish isolates and with PV-0100 was in line with the new classification of this strain as SCPMV. However, the stronger reaction of the same antiserum with the other Spanish isolate indicates a serological variability among the Spanish isolates.

Analysis at the molecular level revealed the presence of a dsRNA molecule of 4.1 kb, an expected size for sobemoviruses, exclusively in SBMV-infected plants of *P. vulgaris*. The additional dsRNA molecule of 20 kb found in these infected plants was also present in healthy plants. Such large linear dsRNAs called 'endogeneous' dsRNAs, have been frequently identified in healthy plants including common bean (Wakarchuk and Hamilton, 1985). These however, are not associated with distinct virus-like particles (Zabalgogeazcoa and Gildow, 1992). Sequencing and analysis of the 810-bp fragment of the ORF2 showed highest homologies with isolates of SBMV. This provides further evidence that the Spanish virus isolates from bean should be regarded as SBMV.

In all symptomatic samples tested, only SBMV was detected. Moreover, for isolate PD21005561, inoculation of single local lesions from *P. vulgaris* 'Pinto' evoked symptoms similar to those observed in Spanish greenhouses. Therefore, SBMV appears to be the causal agent of the new disease in the south-east of Spain.

The identification of SBMV in common bean represents the first report of this virus in Spain. The introduction and dissemination of the virus can be understood through its ways of transmission. SBMV is reported to be seed-borne, although there are contradictory reports as whether it is transmitted to the embryo (Tremaine and Hamilton, 1983). Therefore, the first infections of SBMV in the Spanish greenhouses most probably were introduced via seeds from infected plants. Because SBMV is a very stable virus that is readily transmitted mechanically (Tremaine and Hamilton, 1983), subsequent spread of the virus could occur. The fact that the disease was found to spread along the rows, already early in the season, indicates that mechanical transmission indeed played an important role. This could either be transmission by contact during crop handling or via plant roots in infested soil (Teakle, 1986). The latter mechanisms indeed have been shown to be capable of transmitting SBMV-ALM in *P. vulgaris* 'Donna' (unpublished data). Although beetles of the family *Chrysomelidae* have also been reported to transmit SBMV (Walters, 1969), no indications for virus transmission by beetles were obtained in the south-east of Spain.

Yield losses caused by SBMV can reach 50% as a result of a reduced number and size of seeds (Morales and Castaño, 1985). Nevertheless, in tropical countries the importance of the disease occasionally has been underestimated because symptoms are not very

pronounced and generally the number of pods is not affected. In the south-east of Spain, however, the reduced quality of fresh pods led to considerable economic losses, which constitutes a new feature on the importance of SBMV in the commercial cultivation of common bean. As common bean is an important crop in this area further knowledge on the epidemiology of the virus, e.g. the role of soil in transmission and/or survival of the virus, is of interest for the development of management strategies.

Acknowledgements

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