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RESEARCH PAPERS

Viruses affecting lentil (*Lens culinaris* Medik.) in Greece; incidence and genetic variability of *Bean leafroll virus* and *Pea enation mosaic virus*

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Summary. In Greece, lentil (*Lens culinaris* Medik.) crops are mainly established with non-certified seeds of local landraces, implying high risks for seed transmitted diseases. During April and May of the 2007–2012 growing seasons, surveys were conducted in eight regions of Greece (Attiki, Evros, Fthiotida, Korinthos, Kozani, Larissa, Lefkada and Viotia) to monitor virus incidence in lentil fields. A total of 1216 lentil samples, from plants exhibiting symptoms suggestive of virus infection, were analyzed from 2007 to 2009, using tissue-blot immunoassays (TBIA). *Pea seed-borne mosaic virus* (PSbMV) overall incidence was 4.9%, followed by *Alfalfa mosaic virus* (AMV) (2.4%) and *Bean yellow mosaic virus* (BYMV) (1.0%). When 274 of the samples were tested for the presence of luteoviruses, 38.8% were infected with *Bean leafroll virus* (BLRV). Since BLRV was not identified in the majority of the samples collected from 2007 to 2009, representative symptomatic plants (360 samples) were collected in further surveys performed from 2010 to 2012 and tested by ELISA. Two viruses prevailed in those samples: BLRV (36.1%) was associated with stunting, yellowing, and reddening symptoms and *Pea enation mosaic virus-1* (PEMV-1) (35.0%) was associated with mosaic and mottling symptoms. PSbMV (2.2%), AMV (2.2%), BYMV (3.9%) and CMV (2.8%) were also detected. When the molecular variability was analyzed for representative isolates, collected from the main Greek lentil production areas, five BLRV isolates showed 95% identity for the coat protein (CP) gene and 99% for the 3' end region. Three Greek PEMV isolates co-clustered with an isolate from Germany when their CP sequence was compared with isolates with no mutation in the aphid transmission gene. Overall, limited genetic variability was detected among Greek isolates of BLRV and PEMV.

Key words: legume viruses, RT-PCR, seed-borne viruses, AMV, CMV, BYMV, PSbMV, BLRV, PEMV.

Introduction

Lentil (*Lens culinaris* Medikus) is a traditional Greek cool-season annual crop. Identified from the Upper Palaeolithic period in the Franchthi Cave in Peloponnese (South Greece), it is among the earliest domesticated crops to appear in the country (Hansen and Renfrew, 1978). In recent times, a significant number of lentil 'landraces' highly adapted to local conditions have been cultivated under low input conditions. However, in the era of intensive agriculture their cultivation has been confined to small farms. Increasing international demand and the reformation of the European Common Agricultural Policy have revived interest in legume crops. Today, lentil is cultivated in Greece in about 5000 ha, and domestic consumption is largely deficient (Greek Ministry of Rural and Agricultural Affairs, 2014).

At present, cultivation of local landraces remains unfeasible due to their erratic and low yields. Systematic breeding has been carried out only recently,

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aiming to exploit Greek landraces to develop high-yielding pure-line varieties (Kargiotidou *et al*., 2013). Conservation of landraces *in situ* by farmers, the keystone of their natural adaptation, increases the risk of perpetuating seed-borne viruses (Kargiotidou *et al*., 2015). Five viruses known to be seed-borne in lentil are expected to have high impacts on landrace production. These are the aphid transmitted *Alfalfa mosaic virus* (AMV, genus *Alfamovirus*, family *Bromoviridae*), *Bean yellow mosaic virus* (BYMV, genus *Potyvirus*, family *Potyviridae*), *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family *Bromoviridae*), *Pea seed-borne mosaic virus* (PSbMV, genus *Potyvirus*, family *Potyviridae*), and the beetle transmitted *Broad bean stain virus* (BBSV, genus *Comovirus*, family *Bromoviridae*) (Kumari *et al*., 2009).

At least 30 viruses, including seed-transmitted viruses, can naturally infect lentil. The most important viruses are those associated with yellowing and stunting symptoms, such as *Bean leafroll virus* (BLRV, genus *Luteovirus*, family *Luteoviridae*), or mosaic and mottling symptoms, such as *Pea enation mosaic virus-1* (PEMV-1, genus *Enamovirus*, family *Luteoviridae*) (Kumari *et al*., 2009). Large-scale surveys performed in all legume producing countries have reported extensive spread of BLRV and PEMV-1 (Makkouk *et al*., 2014), resulting in efforts to evaluate their genetic diversity (Vemulapati *et al*., 2010; Šafářová and Navrátil, 2014).

The genome of the phloem-restricted BLRV (5960 nt) (Domier *et al*., 2002), has a structure and function typical for the genus *Luteovirus*, and is organized in six ORFs with respective products named P1 to P6 expressed from the viral genome and three sg mRNAs (Mayo and Ziegler-Graff, 1996). The products of ORF-1 and its overlapping ORF-2 are predicted to produce the viral replicase, ORF-3 encodes the major coat protein (P3 or CP) and ORF-4 codes for the movement protein (P4 or MP) that is nested in the CP coding sequence. The protein encoded by ORF-5 is translated by a read-through of the ORF-3 stop codon, and it is the minor structural subunit also involved in aphid transmission (P3-P5 read-through protein) (Demler *et al*., 1997; Gray and Gildow, 2003; Peter *et al*., 2008). On the other hand, the bipartite genome of PEMV represents the symbiotic association of two taxonomically unrelated ssRNAs or viruses known as RNA-1 (5706 nt; PEMV-1, genus *Enamovirus*) and RNA-2 (4253 nt; PEMV-2, genus *Umbravirus*). Although they are replicating autonomously, encapsidation and aphid-mediated transmission is dependent on the enamovirus PEMV-1 that codes for the major CP and the minor structural, read-through protein (Demler and de Zoeten, 1991; Demler *et al*., 1994). The umbravirus PEMV-2 dictates systemic movement within host plants and mechanical transmission (Demler *et al*., 1993, 1997). Gene organization of PEMV-1 resembles that of the *Luteovirus* genus, except for the absence of OFR-4, and the presence of another ORF (ORF-0) that encodes a silencing suppressor (P0) (Demler and de Zoeten, 1991; Hull, 2002).

Understanding virus epidemiology is a prerequisite to deployment of optimum control measures (Jones, 2004a). As a first step in this direction, the present study aimed to collect missing information on major viruses infecting Greek lentil crops. Using serological techniques, surveys were initially focused on the incidence of the seed-borne AMV, BBSV, BYMV, CMV and PSbMV directly affecting the quality of the local lentil landraces cultivated throughout the country. Our analyses further included BLRV and PEMV that appeared to be the causal agents of the main viral diseases encountered in the fields and the major viruses affecting breeding programmes (Chatzivassiliou *et al*., 2015). A molecular diagnostic tool was developed for their detection using RT-PCR and analysis of their genetic variability. In this study, the first genomic sequence information was obtained for Greek lentil isolates of PEMV and BLRV.

Materials and methods

Lentil field survey

Surveys were conducted from 2007 to 2012 growing seasons to identify viruses affecting lentil crops in the Greek regions of Evros (Orestiada), Fthiotida (Tithorea), Korinthos (Feneos), Kozani (Ptolemaida, Tsotyli), Larissa (Chalkiades, Farsala), Lefkada (Egglouvi), and Viotia (Thiva). Samples were also collected from experimental fields in the farm of the Agricultural University of Athens (AUA, Attiki) (Figure 1). Most crops were surveyed at the flowering-podding growth stage, except where otherwise stated. Incidence of plants with symptoms suggestive of virus infection (stunting, yellowing, reddening, mottling and/or mosaic) was recorded, and ten such samples were collected from each crop. In total, 1576 samples were obtained and analyzed for virus

Figure 1. Surveyed areas of lentil fields in Greece.

presence; 1216 during 2007 to 2009 and 360 from 2010 to 2012.

Virus incidence

A total of 1216 samples collected during 2007 to 2009 were tested for the presence of the seed-borne viruses AMV, BBSV, BYMV, CMV and PSbMV using tissue-blot immunoassays (TBIA) (Makkouk & Kumari, 1996). Nitrocellulose membranes (CN. 0.45 um) (AppliChem, GmbH) were used for the assays, with a battery of polyclonal antibodies provided by the Virology Laboratory, ICARDA, Aleppo, Syria. A number of these samples (274) were also tested against a monoclonal antibody of *Faba bean necrotic yellows virus* (FBNYV, genus *Nanovirus*, family *Nanoviridae)* (3-2E9; Franz *et al*., 1996). The possible presence of luteoviruses was detected by a broad-spectrum monoclonal antibody (5G4; Katul, 1992), while positive samples were further tested against specific monoclonal antibodies for BLRV (4B10; Katul, 1992) and for *Chickpea chlorotic stunt virus* (CpCSV, genus *Polerovirus*, family *Luteoviridae*). A mixture of three monoclonal antibodies (1-1G5, 1-3H4, and 1-4B12) produced against an Ethiopian isolate of CpCSV, and a mixture of three monoclonal antibodies (5-2B8, 5-3D5 and 5-5B8) produced against a Syrian isolate of CpCSV were also used (Abraham *et al.*, 2009).

An additional 360 samples were collected during 2010 to 2012 for further molecular analyses. These were analyzed by enzyme linked immunosorbent assay (ELISA). Samples were tested using monoclonal antibodies for BLRV (1280; Neogen Europe Ltd.) or polyclonal antibodies for CMV (SRA44501; Agdia Elkhart) and AMV (07001), BBSV (07013), BYMV (07007), PSbMV (07030) or PEMV-1 (07029) (LOEWE Biochemica GmbH). Tests were conducted according to the manufacturers' procedures. Five isolates of BLRV and three of PEMV representing the surveyed areas were included in the molecular analysis stud-

ies (Table 1). Original material was maintained at -80°C for RNA extraction and subsequent molecular analyses.

RT-PCR amplification and sequencing of amplicons

Primer design. The PrimerQuest tool (Integrated DNA Technologies Inc.) was used to design primers amplifying almost the entire CP gene (3050-3612 nt, BLRV-CP primers), and the 5081-5697 nt region (BLRV-3′ primers) of BLRV (Table 2, Figure 2) using the full nucleotide sequence of the BLRV genome (BLRV-Michigan: NC_003369.1 = AF441393.1) (Domier *et al*., 2002). For PEMV-1, an internal region (4218-5218 nt) of the P3-P5 read-through structural protein (CP-RTD primers) and the read-through domain (RTD) cited as AT in GenBank (4665-5454 nt, AT primers) were targeted (Table 2, Figure 2), with the full PEMV-1 sequences (PEMV-1-WSG: NC_003629.1) (Demler and de Zoeten, 1991) used as references. Primers were further validated *in silico* in terms of target specificity, using the Primer-BLAST tool of NCBI for each virus against the viral (taxid:10239) and plant (taxid:3193) NCBI database. They were specific only for the indented viral target sequences. A primer set targeting the 18S gene of lentil was also designed to assess the quality of the synthesized cDNA (Giakountis *et al*., 2015).

Total RNA extraction, DNase treatment and cDNA synthesis

Total RNA was extracted from approximately 0.1 mg of infected plant material using 1 mL TRI reagent (MRC) according to manufacturer's protocol. Extracted RNA was quantified with a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific Inc.) and its integrity was visually examined in 1.5% agarose gels. Total RNA (10–15 μg) was treated with 10 U of DNase I (New England Biolabs) for 1 h at 37°C followed by standard phenol:chloroform extraction and ethanol precipitation. Subsequently, 1 μg of DNase treated RNA was used for cDNA synthesis using random primers (Invitrogen) and 200U M-MLV reverse transcriptase (Life Technologies).

Table 1. Listing and geographical origin of lentil *Bean leafroll virus* (BLRV) and *Pea enation mosaic virus* (PEMV) isolates characterized in this study, and sequences deposited at GenBank.

Virus	Isolate	Geographic origin	Target Gene	Accession No.
BLRV	Gr-7	Korinthos (Feneos)	CP	KT382811
			3′ end region	KT382810
	Gr-8	Evros (Orestiada)	CP	KT382812
			3′ end region	KT382809
	Gr-11	Athens (AUA)	CP	KT382813
			3′ end region	KT382808
	Gr-12	Athens (AUA)	CP	KT382814
			3′ end region	KT382807
	Gr-16	Larissa (Chalkiades)	CP	KT382815
			3′ end region	
PEMV	Psi-118	Athens (AUA)	CP-RTD	KT382801
			ORF5 (RTD or AT)	KT382804
	Psi-147	Korinthos (Feneos)	CP-RTD	KT382802
			ORF5 (RTD or AT)	KT382805
	Psi-182	Thessaloniki	CP-RTD	KT382800
			ORF5 (RTD or AT)	KT382803

Table 2. Sequences of primers used in PCRs for the detection and characterization of Greek lentil isolates of *Bean leafroll virus* (BLRV) and *Pea enation mosaic virus* (PEMV).

Primer name	Sequence 5'–3'	Position on genome (bp)	Amplicon size	Target region
BLRV-CP forward	TGTCGTTCGACAATTACAGACC	3050-3612	562	Internal BLRV CP
BLRV-CP reverse	AGGATTCTGTGTCAATACCTTGATAG			
BLRV-3′ forward	GAGTGGAGTAGACCAGATTATGAGC	5081-5697	636	BLRV 3′ end
BLRV-3′ reverse	AACAATCATACCTGTTTTTGTTTCC			
PEMV-CP-RTD forward	CCTCCGATTGCCAGTGTATAA	4218-5218	1000	Internal PEMV CP-RTD
PEMV-CP-RTD reverse	TTCTCGGTATATCCACCATAGGA			
PEMV-AT forward	CTATGAAGGCGTACCCGAATC	4665-5454	789	Internal PEMV RTD (ORF5)
PEMV-AT reverse	TGTAGATCCCACTTGGGTAGT			
18S forward	AAACGGCTACCACATCCAAG	-	250	18S lentil*
18S reverse	CCCATCCCAAGGTTCAACTA			

* Giakountis *et al.*, 2015.

cDNA (1 μg) from an equamolar pool of all RNA samples was used as a negative no RT control.

Polymerase Chain Reaction (PCR) and electrophoresis

PCR annealing temperature and concentration of $MgCl_2$ were optimised for each primer pair. Reactions were performed in 20 μL final volume of a mixture containing 1 μL of cDNA template and 2.5 U of Taq polymerase (Invitrogen) in the presence of 1.5 mM $MgCl_2$. Samples with PCR mix without any template were also included as extra no-DNA controls, together with the no-RT samples. PCR conditions were the following: initial denaturation at 95°C for 4 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 60ºC for 20 sec and extension at 72ºC for 30 sec (for BLRV primers) to 1 min (for PEMV-1 primers), followed by a final elongation step of 72ºC for 5 min. PCR products were analysed in 1.5% standard agarose gels by electrophoresis and inspected under UV after ethidium bromide staining. Positive bands were gel extracted (Purelink Gel extraction kit, Life Technologies) and eluted for direct PCR sequencing.

Sequencing and phylogenetic analyses

PCR sequencing reactions were performed in CEMIA (http://cemia.eu/contact.html), both with forward and reverse primers for each amplicon. Chromatographs were inspected with DNAStar software (http://www.dnastar.com), and for each amplicon both the forward and reverse sequences were aligned with the CLC main workbench 7 software (http://www.clcbio.com/products/clc-main-workbench) to correct for false positive base mismatches due to sequencing errors. Phylogenetic analyses were performed with CLC software using the Neighbor Joining method with Jukes-Cantor as the nucleotide distance measure and 1000 rounds of bootstrapping. Trees were visualised with CLC main workbench 7, while percentage identity matrices (PIM) were calculated with CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2). All branches with less than 70% bootstrap support were collapsed to simplify the tree.

Results

Virus incidence in lentil fields

Field infection during the flowering stages of crop growth, as estimated by the number of symptomatic plants, fluctuated between years and regions, and ranged between 10% and 40%. Greater infection rates were observed in 2010 in Tsotyli (Kozani); early in the spring, farmers completely destroyed several crops established in November due to heavy virus infection. No diseased plants were observed in the

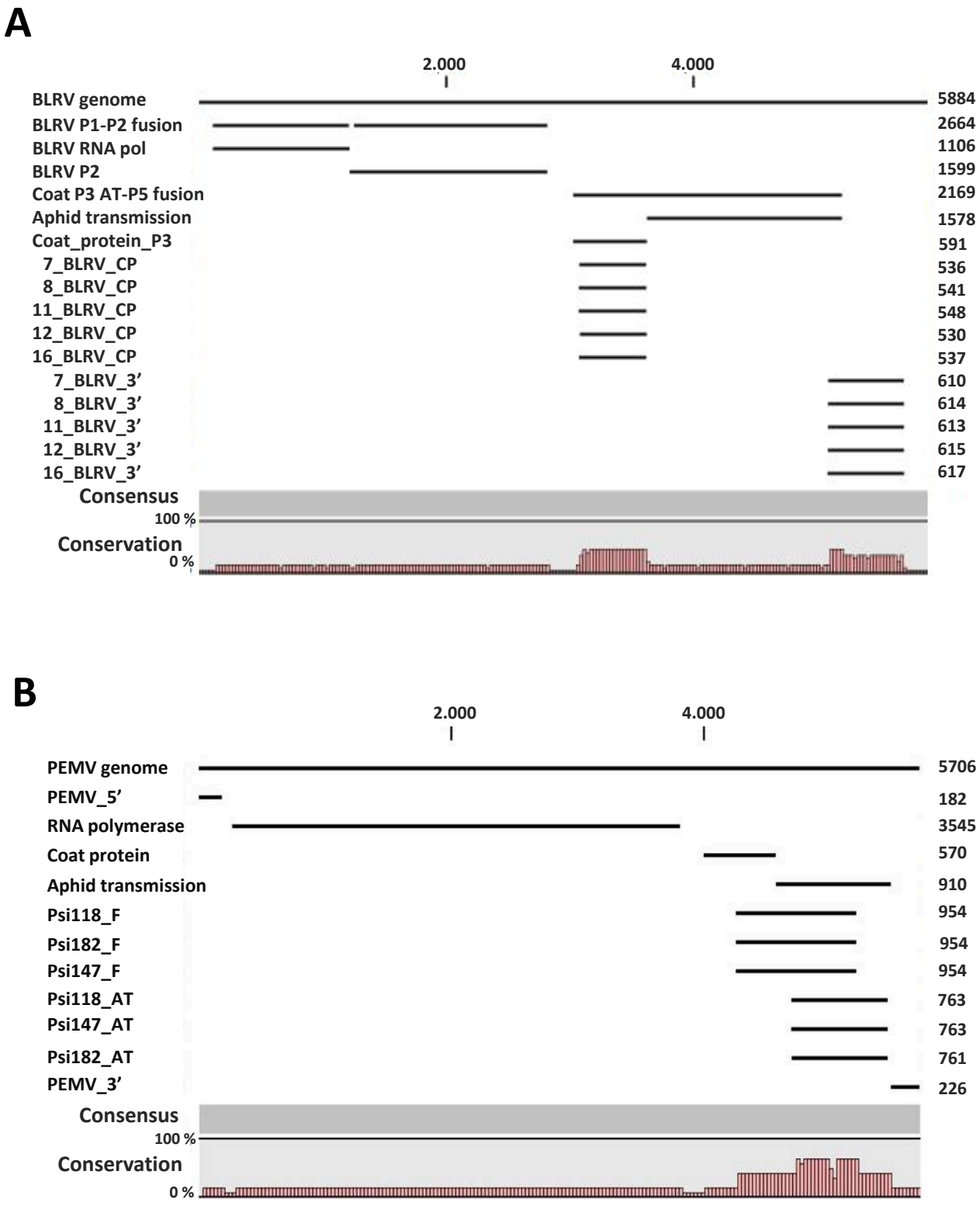

Figure 2. Graphical representation of the selected amplicons for *Bean leafroll virus* (BLRV) (A) and *Pea enation mosaic virus-1* (PEMV-1) (B) against the genome of each virus.

uplands of Egglouvi (Lefkada, in 2007 and 2009) and Thiva (Viotia, in 2008), whereas virus incidence of less than 0.1% was recorded in the uplands of Feneos (Korinthos, in 2012). However, these areas were the only ones surveyed very early in the growing seasons (April) (data not shown).

The presence of seed-borne viruses (their presence in diseased (sampled) plants) varied among cultivated areas, but did not exceed 7.3%, the rate recorded only for PSbMV in Larissa (Chalkiades) in 2008 (Table 3). Few differences were recorded between the two periods of surveys and testing methods. During the 2007 to 2009 surveys, PSbMV was the most common virus with a mean incidence of 4.9% among infected symptomatic plants, followed by AMV (2.4%) and BYMV (1.0%). The seed-borne viruses detected by ELISA during 2010 to 2012 ranked as follows: BYMV (3.9%), PSbMV (2.2%), AMV (2.2%) and CMV (2.8%). For the viruses under study, greater infection rates were recorded during 2010 to 2012 in Larissa for AMV (5.0%), in Kozani for BYMV (10.0%) and in Attiki (AUA) for CMV (6%). BBSV was not detected in any of the samples tested (Table 3).

BLRV was the most frequently detected virus, followed by PEMV-1; their mixed infections were also common (results not shown). Among the 274 samples tested from the 1216 collected during 2007 to 2009, 38.8% were tested positive for luteovirus and BLRV, but not for CpCSV. BLRV was detected in 36.1% of the samples collected during 2010 to 2012. Presence of this virus among infected samples in the different areas fluctuated from 10.0% (Korinthos) to 60.0% (Evros). Plants tested positive for BLRV expressed stunting, yellowing, and reddening symptoms. Tests for PEMV-1 were conducted only during the 2010 to 2012 surveys. This virus was detected in 35.0% of the plants, which usually exhibited mosaic and mottling symptoms. Greater presence among diseased plants was detected for PEMV-1 in Korinthos (80.0%), but this virus was not detected in any sample from Evros and Larissa during the survey periods. FBNYV was not detected in any of the samples tested (Table 3).

Almost 90% of the symptomatic samples collected during 2007 to 2009 tested negative by TBIA for seed-borne viruses. Further tests of selected samples detected BLRV in many diseased plants. Analysis of

Table 3. The mean incidence (%) of viruses detected in lentil crops surveyed in 2007 to 2009 by tissue-blot immunoassay (TBIA), and in 2010 to 2012 by enzyme linked immunosorbent assay (ELISA), in the main lentil-producing areas of Greece.

Region	2007-2009 / TBIA								2010-2012 / ELISA**							
	NS	AMV	BYMV	BLRV	CMV	PEMV	PSbMV	Negative	NS	AMV	BYMV	BLRV	CMV	PEMV	PSbMV	Negative
Attiki	*	*	*	*	*	*	*	*	100	-	2.0	26.0	6.0	58.0	2.0	6.0
Evros	300	2.0	0.7	9.0	-	*	2.7	88.7	20	-	-	60.0	-	-	-	40.0
Fthiotida	60	3.3	-	*	-	*	3.3	93.3	20	-	-	20.0	-	40.0	-	40.0
Kozani	506	4.0	0.8	50.6	-	*	5.6	39.5	120	3.3	10.0	45.0	-	36.7	5.0	0.0
Korinthos	*	*	*	*	*	*	*	*	20	-	-	10.0	-	80.0	-	10.0
Larissa	300	-	2.0	*	-	*	7.3	90.7	80	5.0	-	40.0	5.0	-	-	50.0
Lefkada	50	-	-	*	-	*	-	100.0	*	*	*	*	*	*	*	*
Viotia	20	-	-	*	-	*	-	100.0	*	*	*	*	*	*	*	*
Country	1216	2.4	1.0	38.8	-	*	4.9	61.2	360	2.2	3.9	36.1	2.8	35.0	2.2	17.7

NS = number of samples tested
Negative = % of samples negative to all antisera used
* = no sampling/testing performed, - = not detected,
** All samples were negative to BBSV
Virus acronyms used are: AMV = *Alfalfa mosaic virus*, BBSV = *Broad bean stain virus*, BYMV = *Bean yellow mosaic virus*, BLRV = *Bean leafroll virus*, CMV = *Cucumber mosaic virus*, FBNYV = *Faba bean necrotic yellows virus*, PEMV = *Pea enation mosaic virus-1*, PSbMV = *Pea seed-borne mosaic virus*.

the samples collected during the following years by ELISA suggested a strong association between viral symptoms in the diseased plants and the presence of BLRV and PEMV-1. Nevertheless, no viruses were detected in 17.7% of the tested samples.

Phylogenetic analysis

BLRV primers were designed to amplify the CP gene (described hereafter as CP) and the 3′ end of the viral genome (described as 3′ end, Figure 2, Table 2). PCR amplification with the CP and 3′ end specific primers of BLRV yielded two amplicons, of, respectively, 562 and 636 bp, in the tested isolates. Sequencing of these products resulted in two sequences of 520 and 600 nt average length, after trimming for low quality sequencing calls. Phylogenetic analysis of the Greek BLRV CP sequences revealed that strains Gr-7 (Korinthos) and Gr-16 (Larissa) showed greater genetic variability, out-clustering strains Gr-11 (Athens), Gr-12 (Athens) and Gr-8 (Evros), when only the Greek isolates were compared (Figure 3A). When the analysis was repeated with a collection of BLRV CP sequences from GenBank, the Greek lentil isolates fell within two distinct clusters. Isolates Gr-11 (Athens), Gr-8 (Evros) and Gr-7 (Korinthos) clustered together with previously isolated Greek strains of BLRV from Northern Greece from common vetch (Gr-CV; HE601635.1), bitter vetch (Gr-BV; HE601636.1) and alfalfa (Gr-AA; HE601637.1), with an average nucleotide identity of over 98%. However, isolates Gr-12 (Athens) and Gr-16 (Larissa) clustered independently from the rest of the Greek strains (average nucleotide identity of 92%), and together with strains isolated from alfalfa from Saudi Arabia (Hotah, KJ847770.1; Tabouk, KJ847774.1; Wadi-Edwasser, KJ847775.1). Both strains also showed remote similarity with BLRV strains isolated from faba bean (*Vicia faba* L.) from Australia (Tamworth-1; GQ906583.1) and pea (*Pisum sativum* L.) from Germany (DSMZ; GU002353) (Figure 3C). Analysis of the amplified 3′ end region of the Greek BLRV isolates showed lower diversity (Figure 3B). The average nucleotide identity among Greek isolates was 95% for the CP and 99% for the 3′ end region.

For PEMV, our work focused on RNA-1 of the viral genome and two primer sets against the AT read-through domain were designed. The first domain (described as CP-RTD) targeted the CP sequence at its junction with the rest of the AT gene, while the second (described as AT) targeted the RTD gene downstream of the CP sequence (Figure 2, Table 2). PCR amplification with the CP-RTD and AT specific primers against PEMV amplified two bands of, respectively, 1000 and 789 bp, for all isolates analyzed. Sequencing analysis of all products revealed two sequences of 958 and 760 bp average length, after poor sequencing base correction for all isolates. Phylogenetic analysis of the Greek PEMV isolates for the RTD sequence revealed that all isolates co-clustered with isolates with no mutation in the respective gene (cited as AT gene in GenBank) (Figure 4A). Phylogenetic analysis for the CP-RTD sequence of the same Greek PEMV strains against a collection of PEMV CP sequences from Genbank revealed co-clustering with isolate PEMV-FRG (Z48507) from Germany and the PEMV-SP from the United Kingdom (AF082833.1), but not with the Czech isolate of PEMV UP58 (AY661882.1) (Figure 4B). PIM analysis of the CP sequence (4253-4568 nt) revealed a nucleotide identity of 98% between the German PEMV-FRG strain and the Greek Psi-118 strain, and 90% identity between the same German strain and the Psi-147 and Psi-182 Greek strains.

Discussion

Diseases caused by viruses are threats for legume production worldwide, through adverse effects on seed quality and crop yields. For lentil, economically important viruses differ by production region due to the wide variation of climatic conditions where lentil crops are grown (Kumari *et al*., 1993).

In this first virus survey in lentil crops in Greece, field infections from 0 to 40% were recorded; however, this incidence may sometimes only be indicative. Visual observation usually underestimates virus incidence in lentil (Makkouk *et al*., 2001). Some viruses cause leaf distortion and stunting of the infected plants, which are covered by healthy neighbouring plants after canopy closure (Jones, 2004a; Makkouk and Kumari, 2009). On the other hand, even for surveys performed towards the end of growing seasons, viruses may continue to spread depending on the activity of their insect vectors. Accordingly in our study the lowest incidence of diseased plants was recorded in the areas surveyed very early in the season. Our surveys were technically divided into two time periods (2007 to 2009 and 2010 to 2012), with different serological techniques used (respectively

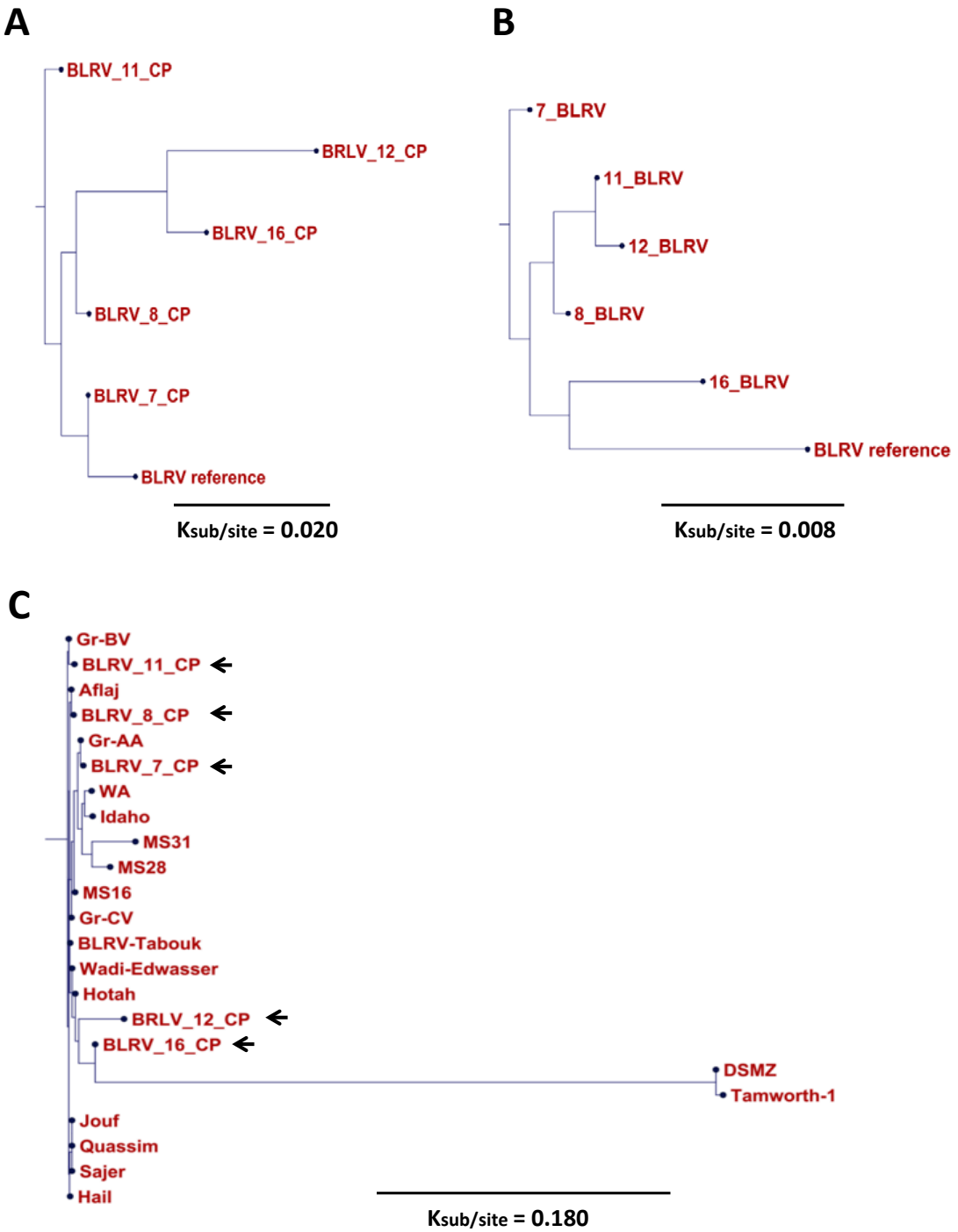

Figure 3. A) Phylogenetic analysis of the *Bean leafroll virus* (BLRV) CP sequences of the Greek isolates, B) Phylogenetic analysis of the 3' untranslated region (UTR) sequences of the Greek isolates, C) Phylogenetic analysis of BLRV CP sequences of the Greek isolates (black arrows) against a collection of BLRV CP sequences from the Genbank: Gr-AA (HE601637.1), Gr-BV (HE601636.1), Gr-CV (HE601635.1), Idaho (GQ404380.1), WA (HM439776.1), Wadi-Edwasser (KJ847775.1), Tabouk (KJ847774.1), Aflaj (KJ847768.1), MS31 (HQ840725.1), MS28 (HQ840724.1), MS16 (HQ840723.1), Hotah (KJ847770.1), DSMZ (GU002353.1), Tamworth-1 (GQ906583.1), Jouf (KJ847771.1), Hail (KJ847769.1), Sajer (KJ847773.1), Quassim (KJ847772.1). Full BLRV sequence (BLRV-Michigan: NC-003369.1 = AF441393.1).

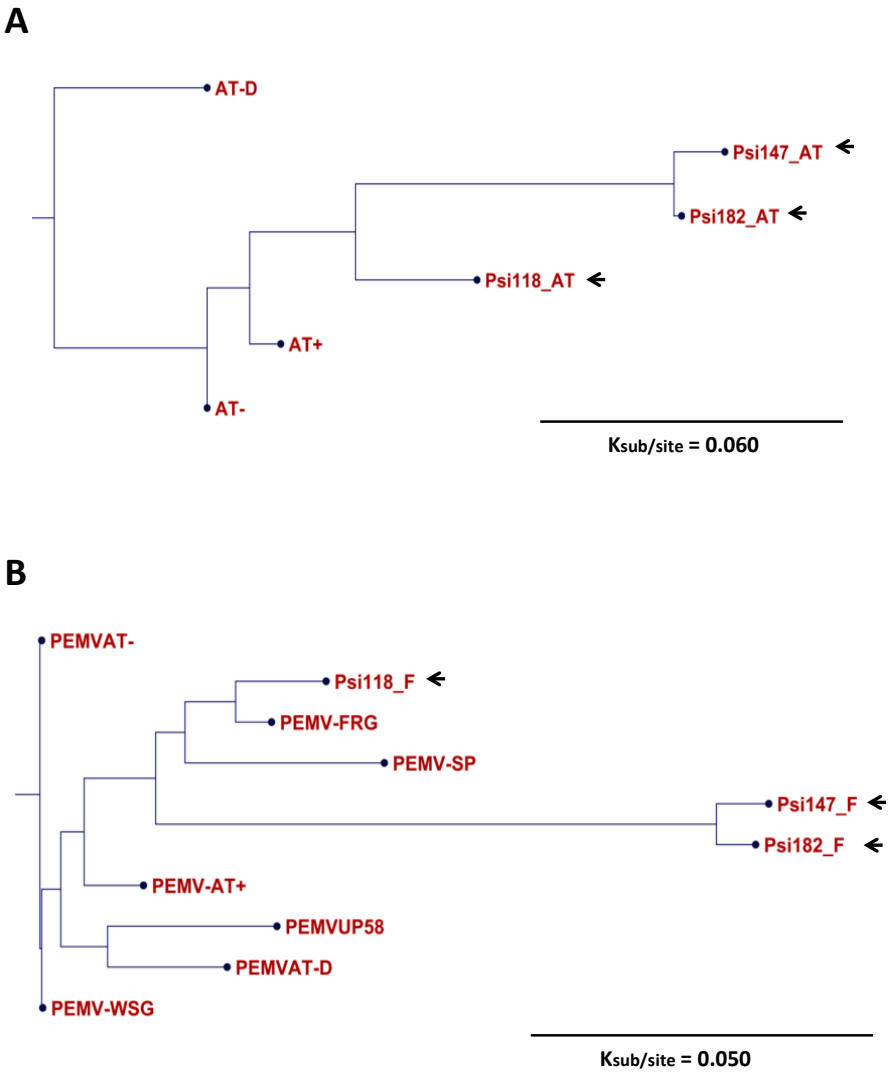

Figure 4. A) Phylogenetic analysis of the *Pea enation mosaic virus* (PEMV) read-through domain (RTD) sequences of the Greek isolates against the respective (AT) sequences of aphid-transmitted strains (AT+) or non-aphid-transmitted strains (AT-D, AT-). B) Phylogenetic analysis of PEMV-CP-RTD sequences of the Greek isolates (black arrows) against a collection of PEMV CP sequences from the Genbank: PEMV-1-WSG (L04573), PEMV-AT- (Y09100), PEMV-AT-D (Y09098), PEMV-UP58 (AY661882), PEMV-FRG (Z48507), PEMV-SP (AF082833), PEMV-AT+ (Y09099).

TBIA and ELISA) during the different periods. However, the relative virus incidences detected did not significantly differ between the two periods.

Based on our surveys, BLRV and PEMV-1 were the most widespread, and these pathogens severely affected all Greek lentil crops. The two viruses were also the most prevalent in the recently bred local landraces (Kargiotidou *et al*., 2013; Chatzivassiliou *et al*., 2015). BLRV is one of the most important plant viruses of legumes worldwide, and PEMV-1 is also considered a significant pathogen in all legume growing regions except Australia (Makkouk *et al*., 2014). Lentil is known to be highly susceptible to BLRV and PEMV-1 (Makkouk and Kumari, 2009), and yield losses of up to 91% (Kaiser *et al*., 1968) and 50% (Kumari *et al*., 2001) have been reported when infection occurred at the pre-flowering crop growth stage. Mixed infections of BLRV and PEMV-1 were

commonly encountered in the present study, and these can be attributed to the common epidemiological features of the two viruses. BLRV and PEMV are non-propagative, aphid-transmitted viruses in the family *Luteoviridae*. They are not known to be seed-borne, but they share narrow, but similar, natural host ranges within the host family *Fabaceae* (Ashby 1984; Skaf and Zoeten, 2000). The pea aphid, *Acyrthosiphon pisum* (Harris), the major species colonizing lentils, and *Myzus persicae* (Sulz.) are considered their main vectors (Cockbain and Costa, 1973; Ashby 1984). The role of perennial pastures such as alfalfa (*Medicago sativa* L. subsp. *sativa*), subterranean clover (*Trifolium subterraneum* L.) or white clover (*T. repens* L.) as major reservoirs for BLRV and PEMV, and their aphid vectors, are crucial for their efficient spread to annual crops (Cockbain and Gibbs, 1973; Damsteegt *et al*., 1995; Jones 2004b; van Leur and Kumari 2011; Peck *et al.*, 2013). In the lentil production areas of Greece, perennial pastures are widespread and no insect control is applied, so large aphid populations develop with subsequent virus spread. Newly established lentil crops may coincide with either late autumn or early spring harvests, and the crops suffer from extended primary BLRV and PEMV infections caused by the dispersing aphid vectors. Additionally, in the absence of insecticide applications, spring weather conditions may also trigger the build-up of large aphid populations in lentil fields, leading to efficient secondary spread of viruses.

In several countries, the presence of non-persistently seed-transmitted viruses may also reach epidemic levels in lentil fields. In Greece, however, incidence of these viruses was low. PSbMV is considered a major viral disease of lentil in some countries in West Asia and North Africa, causing yield losses of up to 73% depending on lentil genotype and virus strain (see Kumari *et al*., 2009). In Greece, PSbMV was detected in pea crops (Chatzivassiliou *et al.*, 2002), and in the non-certified seeds of local lentil landraces (Kargiotidou *et al*., 2015). Recently, an isolate of pathotype 1 was isolated from lentil in fields of the Agricultural University of Athens (Giakountis *et al*., 2015). This virus can be efficiently preserved in lentil crops by seed transmission that ranges from 0 to 44% depending on the genotype (see Kumari *et al*., 2009). Although efficient vectors of PSbMV (Khetarpal and Maury, 1987) are abundant in Greece (Tsitsipis *et al.* 2007), the incidence of this virus was found to be low in the later survey (Giakountis *et al.*, 2015). Identifying factors that influence differences in the spread of PSbMV between years in Greek lentil crops may lead to effective targeted control strategies. Nevertheless, the presence of PSbMV represents a potential risk, especially for the preservation of the Greek lentil landraces (Kargiotidou *et al*., 2015).

Among the viruses considered of minor significance in lentil fields (Kumari *et al*., 2009), AMV and BYMV were detected in several locations in Greece, but their incidence did not exceed 6%. In this country, these viruses can also infect other hosts (Rana and Kyriakopoulou, 1980; Avgelis and Katis, 1989). Their limited spread in lentil may be partially attributed to their low seed transmission rates, that reach 5% for AMV (Jones and Coutts 1996; Makkouk and Attar, 2003) or 0.8% for BYMV (Kumari *et al*., 1994; Jones and Coutts, 1996; McKirdy *et al*., 2000). In some cultivars, however, the yield losses may reach 81-87% due to AMV (Latham *et al*., 2004), or 96% due to BYMV (Kumari *et al*., 1994; McKirdy *et al*., 2000), but their effects on the Greek landraces is unknown. CMV represents another virus that can reduce lentil yields up to 80-90% (Latham *et al*., 2004). The broad host range of CMV and its transmission rate of up to 10% in lentil seeds (Jones and Coutts, 1996; Makkouk and Attar, 2003), and the prevalence of its aphid vectors, are expected to lead to high virus incidence in the field. However, CMV was only occasionally found in the surveyed lentil fields in Greece. BBSV, FBNYV and CpCSV were not detected in our surveys. None of the viruses under study were detected in as great incidence as observed in other similar studies (Makkouk *et al*., 2001). The presence of several other viruses or other lentil disorders (biotic or abiotic stress) (Kumari *et al*., 2009) cannot be excluded as lentil yield-limiting factors.

In general, for the non-persistently transmitted viruses, the most important factors that result in virus epidemics are the close proximity to substantial virus reservoirs and conditions that favour vector reproduction and spread (Jones, 2004a; Makkouk and Kumari, 2009). Infected lentil seedlings early in the season and the proximity of infected perennial crops such as alfalfa may represent major inoculum sources from which various migrating, non-colonizing aphid species can acquire and spread non-persistently transmitted viruses such as AMV and BYMV (McKirdy and Jones, 1995; McKirdy *et al*., 2000). Low incidence of these viruses in the Greek lentil crops may originally reflect late virus introduction in the

crops, resulting in limited virus spread, and low subsequent seed transmission rates. Lentil crops are usually grown in the Greek uplands, where aphid flights are limited or occur only late in the growing season. The fact that none of those viruses was detected in all crops in the uplands of Eglouvi (Lefkada Island), Thiva (Viotia) and Feneos (Korinthos) early in April supports the suggestion that limitation of aphid flights causes low incidence. On the other hand, some Greek landraces may have low intrinsic seed transmission rates or any other types of host resistance, which could be further exploited as genetic traits for their improvement.

In recent years due to the emergence and re-emergence of several vector-borne viruses, the focus has moved toward understanding molecular interaction between plant viruses and their vectors. To better understand the epidemiology of BLRV and PEMV, representing the main viruses threatening lentil crops in Greece, the molecular variability of isolates collected from the main Greek lentil production areas was analyzed. The designed primers targeted the CP gene that is considered as the most conserved region within the *Luteoviridae* family, and is involved in major host and vector interactions (Gray and Gildow, 2003), but it is under the pressure of several selective forces (Torres *et al*., 2005). For BLRV, the phylogenetic analysis of the obtained CP sequences showed that three of our lentil isolates clustered with other Greek isolates from alfalfa, common crops of vetch (*Vicia sativa* L. subsp. *sativa*) and bitter vetch (*Vicia ervilia* (L.) Willd.) (Lotos *et al*., unpublished), further supporting the role of pastures as BLRV sources. On the other hand, limited genetic variability and no geographical or host-associated sub-clustering were detected among our isolates, as two other Greek isolates clustered with strains isolated from alfalfa from Saudi Arabia, faba bean from Australia or pea from Germany. The two isolates from the farm of the Agricultural University of Athens clustered independently within the two groups. For BLRV, when the amplified 610 bp from the 3′ untranslated region (UTR) of the BLRV genome of the Greek isolates was compared with the only available BLRV-Michigan isolate (NC-003369.1 = AF441393.1), this region appeared to be more conserved than the CP sequence. This region controls genome replication and transcription of the *Luteovirus* subgenomic RNAs (Miller *et al*., 2015), and is likely to be highly conserved.

In addition to the major CP gene, viruses of the *Luteoviridae* family encode a second minor read-through structural protein (CP-RTD), which exists as a downstream read-through domain (RTD) fused to the CP gene (Hull, 2002). For PEMV, this 54 kDa CP-RTD protein, and especially changes in the RTD domain, directly interfere with aphid transmissibility (Demler *et al*., 1997). Since only a few sequences are available for PEMV with limited information, no conclusion can be drawn on the basis of their host plants or origins. When analyzing the part of the CP-RTD protein, the Greek lentil PEMV isolates clustered together with PEMV-SP from *Lathyrus odoratus* L. (AF082833.1) from the United Kingdom but not the Czech isolate of PEMV UP58 from *P. sativum* (AY661882.1). Point mutations in the RTD region of PEMV have dramatic effects on aphid transmission (Demler *et al*., 1997), so we specifically amplified the RTD region of the Greek isolates. The respective phylogenetic analysis showed that the isolates clustered with the aphid-transmitted (AT+) genotypes. This does not preclude the presence of non-transmissible genotypes (AT-) within the Greek isolates, as PEMV exists in plants as mixtures of both genotypes whose distribution is the result of numerous selection forces (Demler *et al*., 1997). However, it is expected that AT+ isolates would dominate in the wild type of the virus. In general, limited genetic variability was detected among isolates both of BLRV and PEMV, possibly associated with their restricted host ranges and persistent aphid transmission.

In Greece, if profitability of the lentil crop is to be improved, its cultivation may expand to new environments, and this may increase virus problems. The adoption of efficient integrated management approaches is essential, mainly based on the use of virus-free seeds (Jones, 2004a; Makkouk and Kumari, 2009; Makkouk *et al*., 2014). Toward this goal, more effort should focus on conservation of lentil landraces in order to decrease virus inoculum in seeds (Kargiotidou *et al.*, 2015). Breeding for virus resistance remains the most effective and sustainable virus control method (Makkouk *et al.*, 2014), and is a better alternative to the use of insecticide sprays to control virus vectors. The limited genetic variability of the BLRV and PEMV isolates, and the diversity of the Greek lentil landraces, may represent a promising basis for virus resistance plant breeding programmes.

Literature cited

Abraham A.D., W. Menzel, M. Varrelmann and H.J. Vetten, 2009. Molecular, serological and biological variation among *Chickpea chlorotic stunt virus* isolates from five countries of North Africa and West Asia. *Archives of Virology* 154, 791–799.

Ashby J.W. 1984. *Bean leaf roll virus. CMI/AAB Descriptions of Plant Viruses*, no. 286. 5 pp, Kew, UK.

Avgelis A. and N. Katis, 1989. Identification of *Alfalfa mosaic virus* in Greek alfalfa crops. *Journal of Phytopathology* 125, 231–237.

Chatzivassiliou E.K., H. Boubourakas, S. Winter, D.E. Lesemann, A.D. Avgelis and N.I. Katis, 2002. Viruses infecting pea (*Pisum sativum* L.) crops in Greece. *Phytopathologia Mediterranea* 45, 82.

Chatzivassiliou E.K., E. Ninou, V. Dimitrakas, Aik. Skoufa, Ch. Pankou, A. Lithourgidis, I. Tokatlidis and E.I. Paplomatas, 2015. Spread of aphid-transmitted viruses in honeycomb breeding experimentation in lentil (*Lens culinaris* Medik.). In: *Abstracts 5th Conference of the Interantional Working Group on Legume and Vegetable Viruses,* Haarlem, p. 65.

Cockbain A.J. and A.J. Gibbs, 1973. Host range and overwintering sources of Bean leaf roll and pea enation mosaic viruses in England. *Annals of Applied Biology* 73, 177–187.

Cockbain A.J. and C.L. Costa, 1973. Comparative transmission of Bean leaf roll and pea enation mosaic viruses by aphids. *Annals of Applied Biology* 73, 167–176.

Damsteegt V.D., A.L. Stone and A.D. Hewings, 1995. Soybean dwarf, bean leaf roll and beet western yellows luteoviruses in southeastern US white clover. *Plant Disease* 79, 48–50.

Demler S.A. and G.A. de Zoeten, 1991. The nucleotide sequence and luteovirus-like nature of RNA 1 of an aphid non-transmissible strain of *Pea enation mosaic virus*. *Journal of General Virology* 72, 1819–1834.

Demler S.A., D.G. Rucker and G.A. de Zoeten, 1993. The chimeric nature of the genome of *Pea enation mosaic virus*: the independent replication of RNA2. *Journal of General Virology* 74, 1–14.

Demler S.A., O.N. Borkhsenious, D.G. Rucker and G.A. de Zoeten, 1994. Assessment of the autonomy of replicative and structural functions encoded by the luteo-phase of *Pea enation mosaic virus*. *Journal of General Virology* 75, 997–1007.

Demler S.A., D.G. Rucker-Feeny, J.S. Skaf and G.A. de Zoeten, 1997. Expression and suppression of circulative aphid transmission in *Pea enation mosaic virus*. *Journal of General Virology* 78, 511–523.

Domier L.L., N.K. Mc Coppin, R.C. Larsen and C.J. D'Arcy, 2002. Nucleotide sequence shows that *Bean leafroll virus* has a Luteovirus-like genome organization. *Journal of General Virology* 83, 1791–1798.

Franz A., K.M. Makkouk, L. Katul and H.J. Vetten, 1996. Monoclonal antibodies for the detection and differentiation of faba bean necrotic yellows virus isolates. *Annals of Applied Biology* 128, 255–268.

Giakountis A., A. Skoufa, E. I. Paplomatas, I.S. Tokatlidis and E.K. Chatzivassiliou, 2015. Molecular characterization and phylogenetic analysis of a Greek lentil isolate of *Pea seed-borne mosaic virus*. *Phytoparasitica* 43, 615–628.

Gray S. and F.E. Gildow, 2003. Luteovirus–aphid interactions. *Annual Review of Phytopathology* 41, 539–566.

Greek Ministry of Rural and Agricultural Affairs, 2014. http://www.minagric.gr/images/stories/docs/agrotis/Ospria-Psixanthi/stoixeia_kallierg_ektashospria_2014.pdf (*in Greek*).

Hansen J. and J.M. Renfrew, 1978. Palaeolithic-Neolithic seed remains at Franchthi cave, Greece. *Nature* 71, 349–352.

Hull R. 2002. Family Luteoviridae. In: *Matthews' Plant Virology*, 4th ed. Academic Press, New York, NY, USA.

Jones R.A.C. 2004a. Using epidemiological information to develop effective integrated virus disease management strategies. *Virus Research* 100, 5–30.

Jones R.A.C. 2004b. Occurrence of virus infection in seed stocks and 3 year old pastures of lucerne (*Medicago sativa*). *Australian Journal of Agricultural Research* 55, 757–764.

Jones R.A.C. and B.A. Coutts, 1996. *Alfalfa mosaic* and *Cucumber mosaic virus* infection in chickpea and lentil: incidence and seed transmission. *Annals of Applied Biology* 129, 491–506.

Katul L. 1992. *Characterization by serology and molecular biology of bean leaf roll virus and faba bean necrotic yellows virus*. PhD Thesis, University of Gottingen, Gottingen, Germany, 115 pp.

Kaiser W.J., D. Danesh, M. Okhovat and H. Mossahebi, 1968. Disease of pulse crops (edible legumes) in Iran. *Plant Disease Reporter* 52, 687–691.

Kargiotidou A., E. Chatzivassiliou, C. Tzantarmas, E. Sinapidou, A. Papageorgiou, G.N. Scarakis and I.S. Tokatlidis, 2013. Selection at ultra-low density identifies plants escaping virus infection and leads towards high-performing lentil (*Lens culinaris* L.) varieties. *The Journal of Agricultural Science* 152, 749–758.

Kargiotidou A., E.K. Chatzivassiliou, C. Tzantarmas and I.S. Tokatlidis, 2015. Seed propagation at low density facilitates the selection of healthy plants to produce seeds with a reduced virus load in a lentil landrace. *Seed Science and Technology* 43, 31–39.

Khetarpal R.K. and Y. Maury, 1987. *Pea seed-borne mosaic virus*: a review. *Agronomie* 7, 215–224

Kumari S.G, K.M. Makkouk and I.D. Ismail, 1993. Survey of seed-borne viruses in lentil in Syria and their effects on lentil yield. *Arab Journal of Plant Protection* 11, 28–32.

Kumari S.G., K.M. Makkouk and I.D. Ismail, 1994. Seed transmission and yield loss induced in lentil (*Lens culinaris* Med.) by bean yellow mosaic potyvirus. *LENS Newsletter* 21, 42–44.

Kumari S.G., K.M. Makkouk and B. Bayaa, 2001. *Pea Enation Mosaic Virus-1* infecting lentil in Syria, and further information on its host range, purification, serology and transmission characteristics. *Arab Journal of Plant Protection* 19, 65–72.

Kumari S.G., R. Larsen, K.M. Makkouk and M. Bashir, 2009. Virus diseases and their management. In: *The Lentil: Botany, Production and Uses* (W. Erskine, F. J. Muehlbauer, A. Sarker, B. Sharma, ed.), CABI Publishing, Wallingford, Oxfordshire, UK, 306–325.

Latham L.J., R.A.C. Jones and B.A. Coutts, 2004. Yield losses caused by virus infection in four combinations of non-

persistently aphid-transmitted virus and cool-season crop legume. *Australian Journal of Experimental Agriculture* 44, 57–63.

Makkouk K.M. and S.G. Kumari, 1996. Detection of ten viruses by tissue blot immunoassay (TBIA). *Arab Journal of Plant Protection* 14, 3–9.

Makkouk K.M. and N. Attar, 2003. Seed transmission of *Cucumber mosaic virus* and *Alfalfa mosaic virus* in lentil seeds. *Arab Journal of Plant Protection* 21, 49–52.

Makkouk K.M. and S.G. Kumari, 2009. Epidemiology and integrated management of persistently transmitted aphid-borne viruses of legume and cereal crops in West Asia and North Africa. *Virus Research* 141, 209–218.

Makkouk K.M., M. Bashir, R.A.C. Jones and S.G. Kumari, 2001. Survey for viruses in lentil and chickpea crops in Pakistan. *Journal of Plant Diseases and Protection* 108, 258–268.

Makkouk K.M., S.G. Kumari, J. A.G. van Leur and R.A.C. Jones, 2014. Control of plant virus diseases in cool-season grain legume crops. *Advances in Virus Research* 90, 207–254.

Mayo M.A. and V. Ziegler-Graff, 1996. Molecular biology of luteoviruses. *Advances in Virus Research* 46, 413–460.

McKirdy S.J. and R.A.C Jones, 1995. Bean yellow mosaic potyvirus infection of alternative hosts associated with subterranean clover (*Trifolium subterraneum*) and narrow-leafed lupins (*Lupinus angustifolius*): field screening procedure, relative susceptibility / resistance rankings, seed transmission and persistence between growing seasons. *Australian Journal of Agricultural Research* 46, 135–152.

McKirdy S.J., R.A.C. Jones, L.J. Latham and B.A. Coutts, 2000. Bean yellow mosaic potyvirus infection of alternative annual pasture, forage, and cool season crop legumes: susceptibility, sensitivity, and seed transmission. *Australian Journal of Agricultural Research* 51, 325–346

Miller W.A., J. Jackson and Y. Fenga, 2015. Cis- and trans-regulation of luteovirus gene expression by the 3'end of the viral genome. *Virus Research* 206, 37–45.

Peck D.M., N. Habili, R.M. Nair, J.W. Randles, C.T. de Koning and G.C. Auricht, 2013. *Bean leafroll virus* is widespread in subterranean clover (*Trifolium subterraneum* L.) seed crops and can be persistently transmitted by bluegreen aphid (*Acyrthosiphon kondoi* Shinji). *Crop and Pasture Science* 63, 902–908.

Peter K.A., D. Liang, P. Palukaitis and S.M. Gray, 2008. Small deletions in the *Potato leafroll virus* readthrough protein affect particle morphology, aphid transmission, virus movement and accumulation. *Journal of General Virology* 89, 2037–2045.

Rana G.L. and P.E. Kyriakopoulou, 1980. *Bean yellow mosaic virus* in artichoke in Greece. In: *Proceedings, 5th Congress of the Mediterranean Phytopathological Union*, Patras, Greece, 38–40.

Šafářová D. and M. Navrátil, 2014. Genetic variability of the Czech *Pea enation mosaic virus* isolates. *Czech Journal of Genetics and Plant Breeding* 50, 100–104.

Skaf J.S. and G.A. de Zoeten, 2000. *Pea enation mosaic virus. CMI/AAB Description of Plant Viruses* No. 372.

Torres M.W., R.L. Corrêa and C.G. Schrago, 2005. Analysis of differential selective forces acting on the coat protein (P3) of the plant virus family Luteoviridae. *Genetics and Molecular Research* 4, 790–802.

Tsitsipis J.A., N.I. Katis, J.T. Margaritopoulos, D.P. Lykouressis, A.D. Avgelis, I. Gargalianou, K.D. Zarpas, D.C. Perdikis and A. Papapanayotou, 2007. A contribution to the aphid fauna of Greece. *Bulletin of Instectology* 60, 31–38.

van Leur J.A.G. and S.G. Kumari, 2011. A survey of lucerne in northern New South Wales for viruses of importance to the winter legume industry. *Australasian Plant Pathology* 40, 180–186.

Vemulapati B., K.L. Druffel, S.D. Eigenbrode, A. Karasev and H.R. Pappu, 2010. Molecular characterization of *Pea enation mosaic virus* (genus *Enamovirus*) and *Bean leafroll virus* (genus *Luteovirus*) from the Pacific Northwestern USA. *Archives of Virology* 155, 1713–1715.

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