

CHERRY LEAFROLL VIRUS IN JUGLANS REGIA IN THE LAKE VAN BASIN OF TURKEY

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SUMMARY

Walnut orchards of the Lake Van basin (Turkey) were surveyed from June to October 2006 to determine the incidence of viral infections. ELISA and RT-PCR were used to investigate the presence of *Cherry leaf roll virus* (CLRV) and *Plum pox virus* (PPV), testing a total of 870 samples collected from traditional seed-grown plantations. Whereas no PPV was detected in any of the samples, CLRV was found for the first time in the surveyed locations with an average incidence of 12.9%. Two viral isolates from Edremit were mechanically transmitted to *Chenopodium amaranticolor* in which they caused local chlorotic spots followed by development of small leaves and apical necrosis. A 366 bp DNA fragment was amplified by RT-PCR from the 3' non-coding region of RNA-2 of both viral isolates and sequenced. Isolate Edremit-2 was 93-98% identical to the comparable sequences of other isolates for which information is available, whereas isolate Edremit-1 had a lower sequence identity (53-46%). The size of the coat protein subunits of both viral isolates was 52.4 kDa as determined by electrophoresis.

Key words: walnut, virus, molecular characterization, CLRV, PPV, sequencing, diagnosis.

INTRODUCTION

Walnut (*Juglans regia* L.), is one of the several indigenous fruit tree species of Anatolia with a long history of cultivation (Sen, 1986). Turkey possesses 4.5 million walnut trees with an annual production of 126,000 metric tons, which makes it the fourth major walnut producing country of the world (FAO, 2004; Sutyemez, 2007). Lake Van basin (eastern Anatolia) is currently a prominent walnut production area [nearly 300,000 trees in 1997 (Akca, 2001)], with a prevalence in the provinces of Van and Bitlis.

Cherry leaf roll virus (CLRV), the main viral pathogen of walnut (Civerolo and Mircetich, 2002) is a member of the genus *Nepovirus* but, unlike several of the other species of this genus, is not transmitted by nematodes (Wang *et al.*, 2002). It infects naturally many wild and cultivated woody plants species (Jones, 1986), among which birch (*Betula pendula* Roth), black elderberry (*Sambucus nigra* L.), and sweet cherry (*Prunus avium* L.) (Rebenstorf *et al.*, 2006). More than 30 different walnut cultivars are hosts of this virus but they seldom exhibit recognizable leaf symptoms when plants are ungrafted (Savino *et al.*, 1977; Mircetich *et al.*, 1980; Rowhani and Mircetich, 1992). The walnut strain of CLRV (CLRV-W), which is transmitted naturally by infected pollen, is the causal agent of blackline, an economically important disease affecting English walnuts grafted on black walnuts (*Juglans nigra* L.) (Mircetich and Rowhani, 1984). This virus is widely distributed and has been detected throughout Europe, the former Soviet Union, USA, Chile (Herrera and Madariaga, 2001), New Zealand, Australia, China (Jones, 1986), and Japan (Rebenstorf *et al.*, 2006).

Sharka is one of the most devastating diseases of stone fruits (Dunez and Sutic, 1988; Németh, 1994). *Plum pox virus* (PPV), its causal agent, can infect fruit and ornamental *Prunus* species and other woody hosts. Walnut trees from western Slovakia showing chlorotic spots of the leaves, were reported to react positively to PPV in ELISA and the virus was transmitted from symptomatic leaves to a number of herbaceous hosts (Baumgartnerová, 1996). However, the presence of PPV-infected walnuts trees in Slovakia was not confirmed by later investigations (Polák, 2006).

Since no information was available on virus diseases of walnuts in the Lake Van basin, samples were collected in different areas and tested by ELISA for the presence CLRV and PPV. Only CLRV was detected, its geographical distribution was determined, and two isolates were partially characterized molecularly.

MATERIALS AND METHODS

Field surveys. Samples were collected from commercial orchards and nurseries in the area shown in Fig. 1.

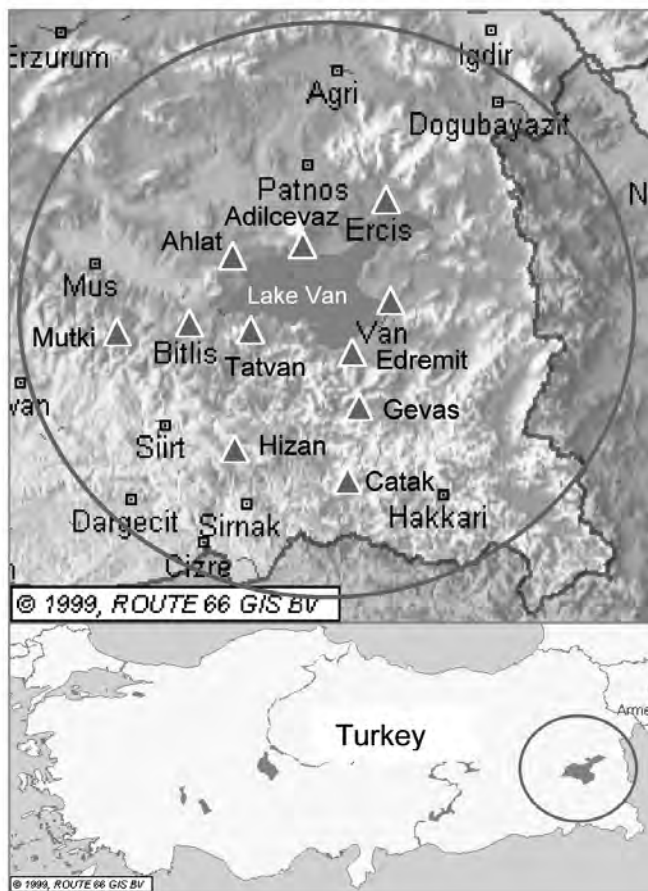


Fig. 1. Area (encircled) in Lake Van basin (eastern Anatolia) surveyed for the presence of walnut-infecting viruses.

A total of 151 orchards were inspected in the province of Van (69) and Bitlis (82). In general, the selection of orchards and collection of samples was related to the relative importance and geographical distribution of the crop. One-year-old twigs were collected from June to October 2006 and stored at 4°C. Leaf tissues were analyzed within a week. A total of 870 samples from symptomatic and symptomless trees were collected from the quadrant of each tree. Crude sap, coat protein (CP) and total RNA were extracted from these leaf samples.

Positive controls. Commercial positive and negative controls (Bioreba, Reinach, Switzerland) for CLRV and PPV were used in serological testing. Two field-grown walnut isolates of CLRV, identified with preliminary tests from the Van province, were also used as positives for CLRV diagnosis. Both isolate were later transferred to young walnut seedlings by chip budding and maintained in a growth chamber at the University of Yuzuncu Yil to serve as virus source during the trials. Leaf tissues of one-year-old shoots of systemically infected *J. regia* were used for ELISA and RT-PCR assays.

Laboratory tests. Serological testing was by DAS-ELISA (Clark and Adams, 1977) using commercial kits

to CLRV and PPV (Bioreba, Reinach, Switzerland). Absorbance values were read at 405 nm using an Awareness Technology microplate reader. Samples were considered positive when the absorbance values at 405 nm exceeded the mean of negative controls by least a factor of two.

RNA preparations for RT-PCR assays were obtained with a modified silica-capture procedure (Foissac *et al.*, 2001). Approximately 100 mg of fresh leaf tissue were placed in a sterile mortar and homogenized with 2 ml of grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc, 25 mM EDTA, 1.0 M KOAc, 2.5% PVP-40, 1% 2-mercaptoethanol). Aliquots of 500 µl of the extract were mixed with 100 µl of 10% sodium lauryl sarcosyl solution in a new set of sterile microfuge tubes and centrifuged at 14,000 rpm with an ALC refrigerated centrifuge for 10 min. Then, 300 µl of the supernatant were transferred to a new eppendorf tube containing 150 µl of ethanol, 100 µl of resuspended silica and 300 µl of 6 M sodium iodide. After centrifugation at 6,000 rpm for 1 min, the supernatant was discarded and the pellet washed twice with washing buffer. The pellet was resuspended with 150 µl of RNase-free water and incubated for 4 min at 70°C, followed by a centrifugation at 14,000 rpm for 3 min. The supernatant was transferred to a new sterile microfuge tube and stored at -20°C until use. Reverse transcription was made as described by Usta *et al.* (2005). Oligonucleotide primers designed according to Werner *et al.* (1997) on conserved region of the 3'-terminal region of viral RNA-2 (F: 5'-TGGC-GACCGTGTAACGGCA-3' and R: 5'-GTCGGAAA-GATTACGTAAAAGG-3') were used for CLRV detection by RT-PCR. Primer, MgCl₂, reverse transcriptase, *Taq* DNA polymerase, and dNTP concentrations were as reported by Usta *et al.* (2005). Samples were amplified in a ThermoHybaid PX2 thermocycler for 40 cycles, using the following schedule: initial denaturation at 94°C for 2 min, denaturation at 94°C for 15 sec, annealing at 50°C for 30 sec and extension at 68°C for 1 min.

Aliquots of 10 µl PCR products were separated either on 5% polyacrylamide gel (PAGE) in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.0) or on 1.5% agarose gel in TAE buffer (40 mM Tris pH 7.8, 20 mM acetic acid, 2 mM EDTA). Amplicons were visualized by ethidium bromide staining (Sambrook *et al.*, 1989).

Mechanical inoculations. For CLRV transmission to herbaceous hosts (*Chenopodium quinoa*, *C. amaranticolor*, *Cucumis sativus*, *Nicotiana benthamiana* and *N. occidentalis*) young walnut leaves were ground in 0.1 M phosphate buffer pH 7.2, containing 2.5% nicotine (Myrta *et al.*, 1996). Inoculated hosts were grown in a greenhouse at 22-24°C and inspected for symptom expression. Symptomatic plants were tested by RT-PCR.

Sequencing and analysis of RT-PCR products. RT-

PCR products obtained from two CLRV-infected *C. amaranticolor* plants were directly sequenced at the facility of Middle East Technical University (Ankara, Turkey). Sequence analysis was performed with Vector NTI Advance™ software (Invitrogen, Carlsbad, CA, USA).

SDS-PAGE and Western blot analysis. Protein fractionation and Western blot analysis were carried out essentially according to Sambrook *et al.* (1989). Leaf samples were collected from infected and healthy *C. amaranticolor* plants and macerated with precooled mortars and pestles in homogenizing buffer (0.05 M Tris pH 7.8, 0.01 M MgSO₄, % 0.02 3,3'-iminobispropylamine, 0.5 M urea and 0.01 M 2-mercaptoethanol) at a ratio of 1 g to 10 ml of buffer. Proteins were electrotransferred to PVDF-plus membrane (GE Water and Process Technologies, Minnetonka, MN, USA). Polyclonal antiserum kindly supplied from Dr. Rowhani (Foundation Plant Services, University of California, Davis, USA) was used as primary antibody at 1:5000 dilution and goat anti-rabbit antibody alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO, USA) was used as secondary antibody at 1:10,000 dilution. Molecular weight of the CP protein of CLRV was estimated from a calibration curve of Log₁₀ molecular weight of standard proteins.

RESULTS

More than 10,000 trees were individually inspected in the surveyed orchards and nurseries and 870 samples were randomly collected during the surveys. Orchards were fairly homogeneous in terms of age and way of cropping, and the trees were vigorous and apparently free of symptoms. In three orchards, however, a few trees were observed that showed dark green or chlorotic spots on the leaves.

The results of serological tests showed that none of the walnut trees tested was infected by PPV, including the few that showed leaf spotting. By contrast, 112 of the 870 samples (12.9%) gave a clear-cut positive reaction to CLRV. Absorbance values of CLRV positives ranged from 1.178 to 2.988 at 405 nm, depending on

Table 1. Outcome of surveys conducted in different Turkish provinces for the presence of *Cherry leafroll virus*.

Province	District	Orchards surveyed (No.)	Samples collected/infected	Infection rate (%)
VAN	Van-Merkez	14	45/2	4.5
	Ercis	13	53/3	3.7
	Edremit	11	39/5	12.3
	Gevas	17	62/30	48.4
	Catak	14	67/2	3.0
BITLIS	Bitlis-Merkez	12	73/7	9.6
	Adilcevaz	17	160/22	13.8
	Ahlat	15	128/14	10.9
	Tatvan	14	89/14	15.7
	Hizan	12	71/4	5.6
	Mutki	12	83/9	10.8
Total		151	870/112	
Average infection rate				12.9

the sample and antiserum used. Negative samples gave absorbance values of 0.069 to 0.244. Seven samples gave inconsistent ELISA reactions, but all yielded the expected amplified product of 416 bp when tested by RT-PCR (Fig. 2). CLRV incidence was highest in Gevas (48%) and lowest in Catak (3%) (Table 1).

Of the six mechanically inoculated herbaceous hosts only *C. amaranticolor* reacted with clear-cut symptoms 7–20 days after inoculation (chlorotic local leaf spots, small leaf development, and apical necrosis), which were the same for the two virus isolates from Edremit. No infection of herbaceous hosts was obtained when systemically infected *C. amaranticolor* tissue was used as inoculum. In particular, all attempts to transfer both virus isolates from *C. amaranticolor* to *C. quinoa* failed, suggesting that these isolates are biologically different from those studied elsewhere, which infect readily this host (Polak *et al.*, 2004). No systemic symptoms appeared in the leaves of *V. unguiculata* and *C. sativus*, and no positive reaction was obtained with RT-PCR.

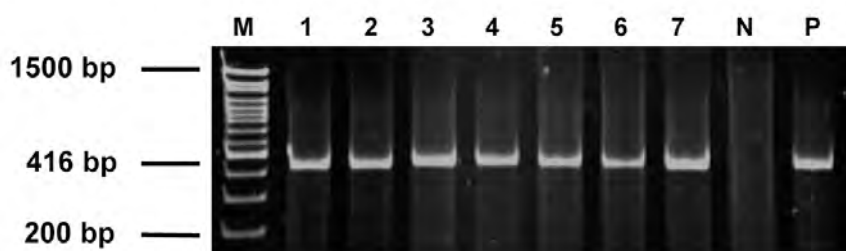


Fig. 2. RT-PCR amplification of a 416 bp fragment using CLRV-specific primers from leaf extracts from walnut trees that gave inconsistent ELISA reactions (lanes 1 to 7). Negative control in lane N; positive control in lane P. Molecular size markers in lane M.

Using total RNA from leaf tissue preparation of two infected Edremit walnut samples as template for RT-PCR, a PCR product of expected size (416 bp) was amplified. The couple of isolates that were transferred to *C. amaranticolor* were also positively identified by RT-PCR. No PCR products were obtained with the healthy leaf tissue controls of *C. amaranticolor* and water blanks.

Sequence analysis and comparison of a 366 bp stretch within the 416 bp fragments amplified from the 3'-terminal non-coding region or RNA-2 of the two CLRV isolates from Edremit (GenBank accession Nos. EF182751 and EF182755) disclosed sequence divergences between CLRV isolates ranging from 43 to 98%. A high variation (up to 45%) was found between the two Turkish isolates. However, CLRV Edremit-2 shared high nucleotide sequence identity (97-98%) with viral isolates from Germany (accession No. AJ877146), UK-1, 2, 3 (AJ877126, AJ877148, AJ877149), Hungary (AJ877150), France (AJ877147, AJ877151), and Slovakia (AJ877152).

Analysis of viral CP extracts by denaturing SDS-PAGE showed the presence of subunits of a single type with a molecular mass of *ca.* 52.4 kDa for both Edremit-1 and Edremit-2 isolates (not shown). However, no clear-cut reactions were obtained in Western blot.

DISCUSSION

This is the first walnut virus survey made in Turkey, which shows that in the Lake Van basin walnuts are infected only by CLRV, thus confirming Polak's (2006) findings on the absence of PPV infection in this species.

Except for a peak of 48% infection in Gevas, CLRV incidence in the surveyed area was lower (3 to 15%) than that generally reported from other European countries. For instance, in southern England CLRV infects about half of mature walnut trees (Cooper, 1980) and birches (*Betula* spp.) can be locally infected to a similar extent (Cooper and Atkinson, 1975; Massalski and Cooper, 1983). Mircetich and Rowhani, (1984) reported natural CLRV spreading in Californian orchards and, the percentage of the infected seedlings was *ca.* 32% in Italy (Quacquarelli and Savino, 1977) and 20% in France (Delbos *et al.*, 1983). CLRV is also widespread in English walnut in Hungary (Kölber *et al.*, 1982). Since the virus is efficiently seed and pollen-transmitted (Civerolo and Mircetich, 2002), it may have been introduced in the Lake Van basin through seeds.

In general, the properties of the two Turkish CLRV isolates presently studied did not seem to differ much from those reported in the literature, except for inability to infect *C. quinoa*. This, however is a minor divergence for other properties, physicochemical and molecular, are basically the same. For instance, CP subunits were 52.4 kDa in size, a figure in agreement with other size estimates that range from 51.5 to 56 kDa (Cooper and

Atkinson, 1975; Gentkow *et al.*, 2005; Pallas *et al.*, 1992; Scott *et al.*, 1992), and the sequence of a 366 bp stretch of the 3'-NCR of RNA-2 of isolate Edremit-2 was highly homologous (albeit not identical) with sequences of comparable regions of other viral isolates from databases. However, a significant level of variability was found within the same genomic stretch between the two Turkish virus isolates, the meaning of which requires further and more extensive molecular investigations.

This is the first report of the occurrence of CLRV in walnut in Turkey a finding that calls the implementation of a sanitary certification programme to provide elite, virus-free propagation material to registered nurseries.

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