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Detection and characterization of stone fruit virus diseases in Tunisia

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Summary. A preliminary survey was conducted in Tunisia to identify stone fruit (almond, plum, peach) virus diseases occurring in orchards and mother block stands. Two ilarviruses, *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) infecting almond and plum and a trichovirus, *Apple chlorotic leaf spot virus* (ACLSV) found in a peach orchard in northern Tunisia, were detected. These viruses were detected by means of herbaceous and woody indicators (cucumber, peach GF 305), serology (DAS-ELISA), electron microscopy (DIP, ISEM, Decoration) and reverse transcription-polymerase chain reaction-based assay (RT-PCR). Furthermore, typing PNRSV isolates was done by bioassays using various species and cultivars of Cucurbitaceae, serotyping with specific monoclonal antibodies (Mabs) in ELISA-DASI and RFLP analysis. Characterization work showed that serotyping with Mabs was the best mean to distinguish between PNRSV isolates.

Key words: virus diseases, stone fruits, Tunisia, detection, characterization.

Introduction

The total area used for fruit cultivation in Tunisia is approximately 470,000 ha, producing mainly citrus, grapevine, stone and pome fruits. Stone fruits are cultivated on some 380,000 ha. Among the stone fruits traditionally grown in Tunisia, almond is the most important and widespread crop (310,000 ha). Peach ranks second with about 22,000 ha, while apricot and plum rank third and fourth, being grown on 17,000 ha and 4,100 ha respectively.

Almond, plum and peach crops are affected by various virus diseases, mainly *Prunus necrotic ringspot* (PNRSV), *Apple mosaic* (ApMV), *Prune dwarf* (PDV), *Apple chlorotic leaf spot* (ACLSV) and *Plum pox* viruses (Canova, 1985; Conti *et al*., 1985; Giunchedi and Poggi Pollini, 1985; Savino, 1985).

The importance of virus and virus-like diseases in stone fruits is little known in Tunisia. Recently, more attention has been paid to these diseases in an effort to improve control methods. Field surveys have been conducted in commercial orchards as well as in mother block stands of the Groupement Obligatoire des Viticulteurs et Producteurs de Fruits (GOVPF) and the Groupement Interprofessionnel des Agrumes et Fruits (GIAF). Investigations have shown that in almond-growing areas, almond mosaic (Dunez, 1986; Boulila, 1992; 1997; Zeramdini *et al*., 1996) and bud failure (Siriez, 1981) are the most common diseases in Tunisia.

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Almond mosaic is characterized by a variety of field symptoms that have long been familiar (Scaramuzzi, 1956, 1957; Ciccarone, 1958). Those studies have led to the identification of some relatively specific syndromes, such as line patterns, yellow mottle, vein banding and yellow speckling (Fig. 1a).

The present paper deals with results on detection and characterization of viruses found infecting almond, plum and peach grown for fruit and propagating material production.

Materials and methods

Field surveys

Investigations were carried out in orchards throughout the country (Sfax, Jemmal, Cap Bon, Mehrine) and in the most important Tunisian mother block stands of stone fruits, especially those of the GIAF (at Sbikha and Oued Mliz) and the GOVPF (at Grombalia).

Sampling

Surveys and sample collection were carried out in early spring (1991–2000). Eighteen almond accessions, one of peach and one of plum were collected. Each accession was made up of 20 shoots (collected from several trees) bearing leaves with virus symptoms and taken from the four compass points and from the internal part of the tree.

Mechanical transmission

Cucumis sativus L. cv. Marketer was inoculated with sap from diseased almond and plum trees by crushing their leaf tissues in 0.1 M phosphate buffer, pH 7.4 containing 0.1% 2-mercaptoethanol and rubbing the extract onto celite-dusted leaves. Inoculated plants were kept in a temperature-controlled glasshouse at 18–24°C.

Grafting

Positive samples were graft-inoculated to 4-month-old GF 305 peach seedlings. After two weeks, the shoot extremity was excised giving rise to axillary buds. Inoculated plants were kept in the greenhouse at the above conditions.

Electron microscopy

Three electron microscopy procedures were used in this work: quick leaf-dip, immunosorbent electron microscopy, using negative staining with uranyl acetate, and decoration. The antisera to PNRSV (titre: 1:512) and to PDV (titre: 1:128) were provided by G. Pasquini and M. Barba from the Istituto Sperimentale per la Patologia Vegetale, Rome, Italy.

Biological indexing

Four PNRSV isolates (Peerless, Achak, Mosaic of Sfax and Chlorosis of Sfax) were mechanically inoculated to herbaceous hosts from various botanical families in order to characterize them biologically. Hosts plants were: Cucurbitaceae (*C. sativus* of the following cultivars: Marketer, Poinsett, Sombre d'Arménie, Grandiose de Reuter; *Cucurbita pepo* cv. Jedida and *Cucurbita maxima* cv. Béjaoui); Leguminosaceae (*Vigna unguiculata*), Chenopodiaceae (*Chenopodium foetidum* and *Chenopodium murale*) and Solanaceae (*Nicotiana tabacum* cv. Xanthi, *N. clevelandii*, *N. rustica*, *N. benthamiana*, *N. glutinosa* and *N. occidentalis*). Trials were conducted at temperatures ranging from 18 to 24°C.

Based on the results of this bioassay (Boulila and Marrakchi, 1999), a second trial was carried out in a climatic chamber with a constant temperature of 22°C and a relative humidity of 50% using a variety of new cultivars of Cucurbitacae available on European markets. Five PNRSV isolates (Peerless, Ferraduel, Ksontini, Fournat de Brezenaud, Nec+Ultra) detected in Tunisian mother block stands were sap-transmitted to the following plants: *C. sativus* cv. Marketer, Marketmore 70, Miracross F_1, Wisconsin SMR 18, White wonder and Sensation; *Cucumis melo* cv. Tendral verde, Hale's Best and Planter's Jumbo; *C. pepo* cv. Diamant F_1, Greyzini F_1 and San Pasquale; an ornamental *Cucurbita* sp., and *C. maxima* cv. Béjaoui and Butternut.

ELISA

The direct double-antibody sandwich method of an ELISA kit supplied by Sanofi Pasteur (Marnes-La-Coquette, France) was used following the procedure of Clark and Adams (1977). The naturally (almond, plum and peach) and artificially (cucumber) infected materials were tested for PNRSV, PDV, ApMV and ACLSV.

Three isolates of PNRSV (Peerless, Texas and Khoukhi) from naturally infected almond and experimentally infected cucumber were included in

a serotyping study using nine monoclonal antibodies (Mabs) in ELISA-DASI following the procedure of Boscia *et al*. (2000) with minor modifications. Nine Mabs (41, 236, 242, 294, 348, 399, 460, 503 and 563) provided by D. Boscia (CNR, Bari, Italy) were used. Almond leaves were ground with 7 ml extraction buffer (only 4 ml for cucumber leaves). The positive control (peach GF 305 cuttings infected with PNRSV) was provided by A. Minafra (CNR, Bari, Italy), and the negative control was supplied by Sanofi Pasteur.

Total nucleic acids extraction, cDNA sythesis, PCR and RFLP

One hundred mg tissue was extracted with 1 ml buffer consisting of 1 ml 10x EB (500 ml of a mother solution made up of 38.5 g glycine, 29 g NaCl and 100 ml 0.5 M EDTA, pH 8), 2 ml 10% SDS, 1 ml 10% N-Lauroyl Sarcosine and 6 ml bidistilled water. Only 400 µl of extracted material was added to 800 µl of a phenol:chloroform:isoamylic alcohol (25:24:1) solution. Vortexing and centrifugation (500 *g*, 10 min.) were applied and two volumes of phenol:chloroform:isoamylic alcohol (25:24:1) were added. One volume of chloroform:isoamylic alcohol (24:1) was added. After vortexing and centrifuging 1/5 volume of ammonium acetate 10 M and 3 volumes of absolute ethanol were added, the solution was vortexed again and allowed to stand at -70°C for at least 20 min. After ethanol-precipitation, a vortex and a centrifugation were done and pellets were thoroughly washed with 70% ethanol. After additional centrifugation pellets were vacuum-dried and resuspended in 50 µl sterile water. Tubes were stored at -70°C.

CDNA was synthesized following the procedure by Hadidi *et al*. (1995).

To 5 µl of cDNA were added: 14 pmoles of the upstream primer (5'-AGA CGT CGT GAC AGA CGT CGA AG-3'), 18.3 pmoles of the downstream primer (5'-CTT CGG ACC ATA GAC ATC-3') synthesized at M-Medical-Genenco (Florence, Italy) in the sequence of PNRSV RNA-3 given by Hammond and Crosslin (1995) (Table 1); 5 µl 10x RT buffer [Sigma (St. Louis, MI, USA), 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin]; 1 µl *Taq* DNA polymerase (5U/µl, Sigma) and 36 µl sterile water. PCR was performed using a thermal cycler (Omn-E, Thermo Hybaid, London, UK) for 1 cycle at 85°C for 5 min. (denaturation), 30 cycles at 94°C for 30 s (denaturation), 50°C for 30 s (annealing), 72°C for 45 s (extension), with a final extension at 72°C for 5 min (1 cycle) (Rowhani *et al*., 1995; Crescenzi *et al*., 1997).

PCR-amplified fragment analysis was carried out using 6% polyacrylamide silver staining gel electrophoresis adapted to the Protean II xi cell

Table 1. Size and position of amplified fragment of *Prunus necrotic ringspot virus* (PNRSV) isolates and corresponding accession numbers (GenBank EMBL).

PNRSV isolate	Amplified fragment size (bp)	Position of the amplified fragment in the genomic RNA-3 of PNRSV isolates	Accession number
CH 61	282	419-700	AF034989
CH 39	282	418-699	AF034990
CH 38	282	419-700	AF034991
CH 9	282	419-700	AF034992
CH 57	282	420-701	AF034993
CH 30	282	414-695	AF034994
CH 71	282	419-700	AF034995
Mission	282	379-660	AF013285
Prune	282	379-660	AF013286
SW 6	282	379-660	AF013287
PE 5	282	531-812	L38823
PV 96	282	530-811	S78312
30/4	282	538-819	U57046
PV 32	282	533-814	Y07568

Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) system, a vertical slab-gel electrophoresis apparatus. The running buffer was 0.5x TBE.

PCR products were subjected to RFLP analysis with *Ava*II and *Nla*III restriction endonucleases (BioLabs Inc., Beverly, MA, USA). An aliquot (3 µl) of the PCR products of the previously mentioned isolates was digested by addition of the following mix: (i) 2.5 µl *Ava*II 1x NE Buffer 4 [50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate and 1mM DTT (pH 7.9 at 25°C)], 0.5 µl *Ava*II, 4 µl twice-distilled water. (ii) *Nla*III: 2 µl 1x NE Buffer 4, 0.5 µl Bovine serum albumine, 0.5 µl *Nla*III, 4 µl bidistilled water. Digestion was done at 37°C for 1 hour as recommended by the manufacturer.

The digested products were electrophoresed in a vertical 6% polyacrylamide gel adapted to the Mini-Protean II (Bio-Rad Laboratories) system.

Results

Field surveys

Almond

In this survey, mosaic syndrome was observed on different almond varieties grown in commercial orchards in all traditional areas of cultivation (Sfax, Menzel Kamel, Ouardanine, Kairouan), on varietal collections of the Institut National de la Recherche Agronomique de Tunisie (INRAT) at Sfax and Mornag, and on twenty-year-old mother blocks for budwood production established at Grombalia by the GOVPF and at Sbikha by the GIAF.

In 1998, in an orchard located in the Sfax region (southern Tunisia), on an unidentified variety, there was a single case of a unilateral chlorosis reminiscent of almond calico from the USA, as reported earlier [Thomas and Rawlins, 1939 (cited in Nemeth, 1986)]. With almond calico, the leaves of infected trees develop extended chlorotic spots. Spots were scattered over the entire leaf except the main vein, or they coalesce at the leaf base or leaf tip. Chlorotic areas are pale yellow or white and are especially conspicuous in spring. A characteristic symptom of this disease is stunting of the flowers and leaf buds. Moreover, fruits are small with a pale yellow skin. The infectious nature of this disease and its relationship to PNRSV was reported by Nyland and Lowe [1964 (in Nemeth, 1986)]. Also in the Sfax region, some trees showed the classical mosaic syndrome.

Another particular symptom was observed in a mother block stand at the GIAF: an extensive pale-green area bordering the main leaf vein. It occurred only on the Tunisian variety "Achak".

Plum

Chlorotic spots and line and oak leaf pattern symptoms were observed on the leaves of plum trees (cv. Methley) at Beni khalled, Nabeul district. The infected trees were in a commercial orchard established by the Office des Terres Domaniales.

Peach

Chlorotic blotches and line patterns on the leaves, usually distributed asymmetrically, as well as stunting of the shoots (Fig. 1b) were observed on peach trees (cv. Boutabgaia) during the spring of 1999.

Mechanical transmission

Chlorotic and necrotic lesions were observed on cotyledons of *C. sativus* cv. Marketer. Systemic symptoms consisted of severe mosaic (Fig. 1c), compact growth and top necrosis. These symptoms were typical of PNRSV infection. However, PDV was recognized when the mosaic was limited to a lateral area of the leaf blade (Fig. 1d), without leaf tip necrosis.

Grafting

Infection with PNRSV was seen on peach GF 305 with chlorotic (Fig. 1e) and necrotic lesions leading to leaf perforation due to dead tissue (Fig. 1f). Such a symptom is called as tatter leaf.

Electron microscopy

PNRSV and PDV particles were visualized by electron microscopy. Virions were trapped, (Fig. 2 and 3) and decorated by the specific antiserum.

Biological indexing

The two Cucurbitaceae (*C. sativus* cv. Marketer and *C. maxima* cv. Béjaoui) reacted differently to the four Tunisian PNRSV isolates placing them in 3 PNRSV subgroups: 1. Mosaic of Sfax and Peerless, 2. Achak and, 3. Chlorosis of Sfax. *V. unguiculata* reacted to PNRSV by forming a chlorotic line pattern and deformation of the leaf lamina. No symptoms were obtained with plants in the

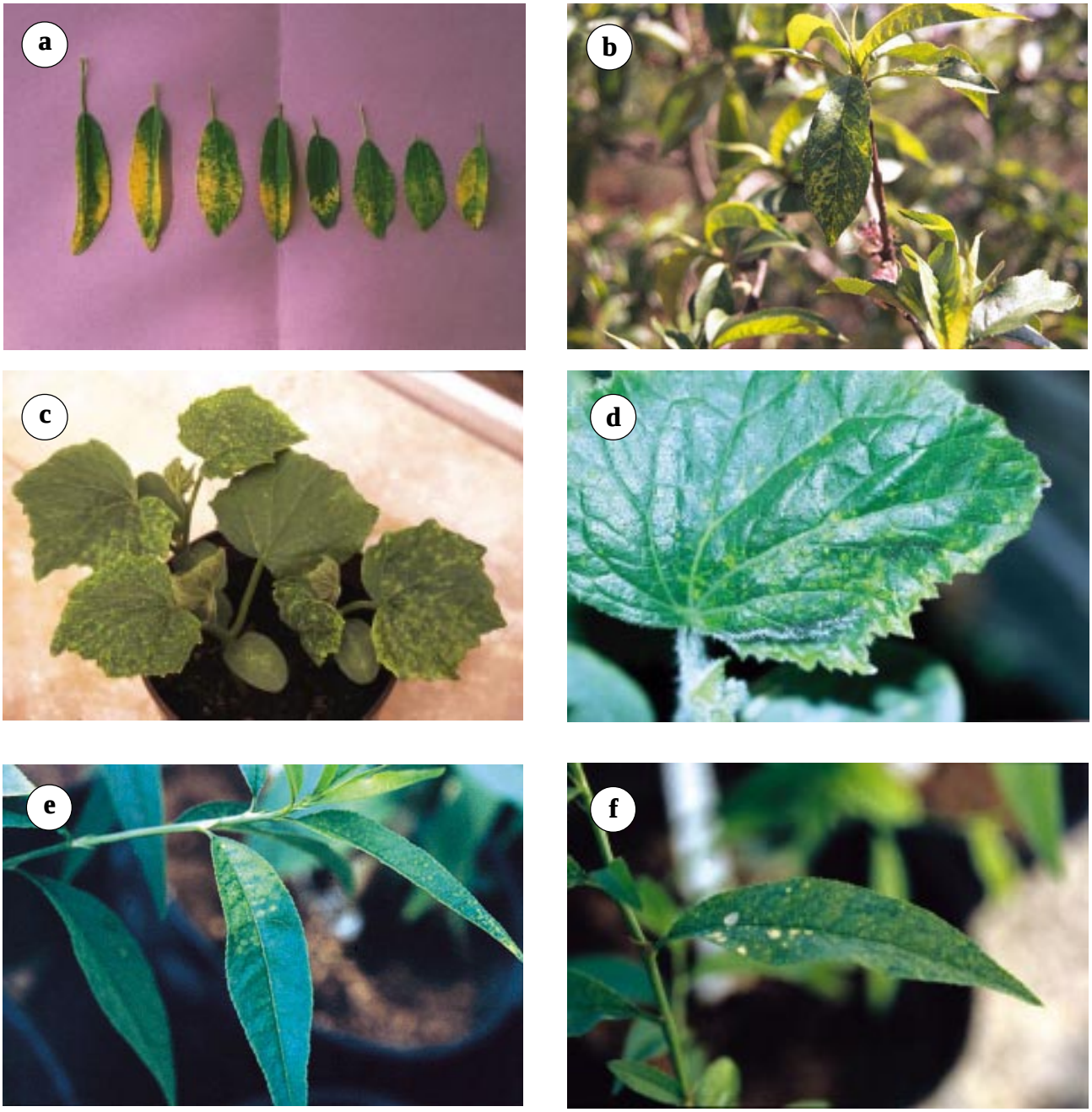

Fig. 1. a. Various forms of mosaic syndrome on almond leaves. b. Chlorotic blotches on Apple chlorotic leaf spot-infected peach leaves. c. Mosaic on true leaves of *Cucumis sativus* cv. Marketer. d. Asymmetrical mosaic of a Prune dwarf virus on a true leaf of *Cucumis sativus* cv. Marketer. e. Chlorotic rings on Prunus necrotic ringspot-infected peach GF 305 leaves. f. Chlorotic and necrotic rings and tatter leaf symptom on peach GF 305.

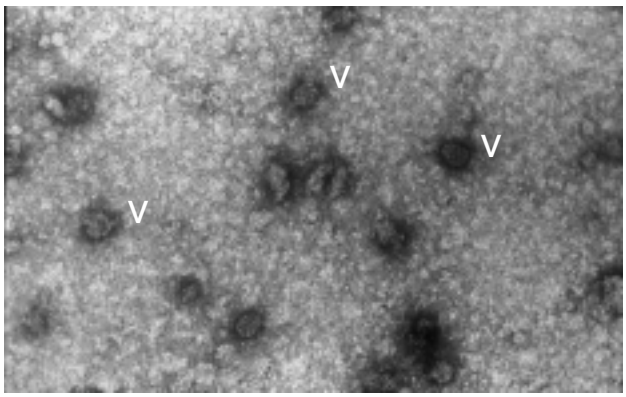

Fig. 2. Prunus necrotic ringspot virus particles using the ISEM technique (Magnification: x160.000); v=virus.

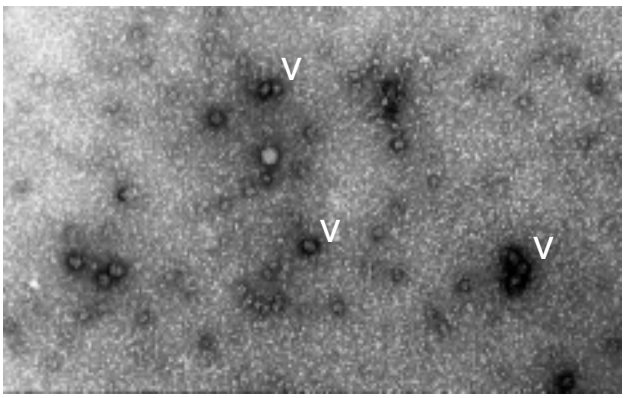

Fig. 3. Prune dwarf virus particles using the ISEM technique (Magnification: x157,500); v=virus;

Chenopodiaceae and Solanaceae families (Table 2). Based on these data, new cultivars were artificially inoculated with pure sources of PNRSV, revealing the existence of two clusters: 1. Ferraduel and Peerless and, 2. Ksontini, Nec+Ultra and Fournat de Brezenaud (Table 3). Table 4 shows how the herbaceous cultivars reacted to PDV infection. The main symptoms consisted of primary chlorotic and

Table 2. Host reactions to infection with 4 Tunisian *Prunus necrotic ringspot virus* isolates (PNRSV): Mosaic of Sfax, Peerless, Achak, Chlorosis of Sfax. Different groups are distinguished by different letter fonts in a column: normal, bold, underlined. No symptom was recorded on any of the Chenopodiaceae and Solanaceae species tested.

Host plant	Cotyledonary leaves				Fully developed leaves			
	Mosaïque de Sfax	Peerless	Achak	Chlorose de Sfax	Mosaïque de Sfax	Peerless	Achak	Chlorose de Sfax
Cucurbitaceae								
Cucumis sativus cv. Marketer	C.Ch..L.	C.Ch..L.	0	C.Ch..L.	Mo, V.B.	Mo, V.B.	Mo,V.C.,U.Mo	Mo, V.B..
Cucumis sativus cv. Poinsett	0	0	0	0	0	0	0	0
Cucumis sativus cv. Sombre d'Arménie	0	0	0	0	0	0	0	0
Cucumis sativus cv. Grandiose de Reuter	0	0	0	0	0	0	0	0
Cucurbita pepo cv. Jedida	0	0	0	0	0	0	0	0
Cucurbita maxima cv. Béjaoui	0	0	0	0	Y.S.	Mo, Y.S.,. C.Ch.L.,R.L	**Mo, M.Ch. N.**	**Y.S. C.Ch.L., U.Mo.**
Leguminosaceae								
Vigna sinensis	0	0	0	0	L.Ch.L., D.	L.Ch.L, D.	L.Ch.L.	L.Ch.L.

Mo: Mosaic, V.C.: veinal chlorosis, U.Mo: unilateral mosaic, M.Ch.: marginal chlorosis, L.Ch.L.: linear chlorotic lesions, C.Ch.L.: circular chlorotic lesions, V.B: vein banding. Y.S.: yellow speckle, R.L: reduced leaf, D.: deformation, N.: necrosis, 0: no symptoms.

Table 3. Host reactions to infections with 5 *Prunus necrotic ringspot virus* isolates (PNRSV). Different groups were detected, which are distinguished by different letter fonts: underlined or not. The virus isolates tested were: Ksontini (PNRSV-1), Ferraduel (PNRSV-2), Peerless (PNRSV-3), Nec+Ultra (PNRSV-4) from the GOVPF stand at Grombalia, and Fournat de Brezenaud (PNRSV-5) from the GIAF stand at Sbikha. Inoculation made on 23.08.99. Observations on 12th day after inoculation.

Host plant	Local reaction					Systemic reation				
	PNRSV-1	PNRSV-2	PNRSV-3	PNRSV-4	PNRSV-5	PNRSV-1	PNRSV-2	PNRSV-3	PNRSV-4	PNRSV-5
Cucumis sativus										
cv. Marketer	0	N.L	N.L.	Ch.L.	0	L.Mo., Dw.	S.Mo., Dw.	S.Mo.,Dw.	L.Mo.,Dw.	L.Mo., Dw.
cv. Miracross F_1	Ch.L.	b.N.L.	b.N.L.	b.N.L.	Ch.L.	Mo, Dw.	S.Mo., m.Dw.	S.Mo., m.Dw.	S.Mo.,m.Dw.	Mo, Dw.
cv Wisconsin SMR 18	Ch.L.	Ch.L	Ch.L.	Ch.L.	L.Ch.	Mo., m.Dw.	V.B., Dw.	V.B., Dw.	Mo., m.Dw.	Mo., m.Dw.
cv. Marketmore 70	0	N.L	N.L.	0	0	Mo., m.Dw.	Mo., m. Dw.	Mo., m.Dw.	Mo., m.Dw.	Mo., m.Dw.
cv. White Wonder	0	N.L.	N.L.	0	0	L.Mo., Dw.	Mo., Dw.	Mo., Dw.	L.Mo., Dw.	L.Mo., Dw.
cv. Sensation	Ch.L.	N.L.	N.L.	Ch.L.	Ch.L.	St. Mo., Dw.	St.Mo., Dw.	St.Mo., Dw.	St.Mo., Dw.	St.Mo., Dw.
Cucumis melo										
cv. Tendral Verde	Ch.N.L.	Ch.N.L.	Ch.N.L.	0	0	Mo., s.Dw.	Mo., s.Dw.	Mo., s.Dw.	L.Mo.	L.Mo.
cv. Hale's Best	N.L.	N.L.	N.L.	N.L.	N.L.	Mo, m.Dw., N.	Mo, m. Dw., N	Mo., s.Dw.	Mo, s.Dw.	Mo., s.Dw.
cv. Planter's Jumbo	0	N.L.	N.L.	0	0	L.Mo.	L.Mo., N.	L.Mo., N.	L.Mo.	L.Mo.
Cucurbita pepo										
cv. Diamant F_1	0	0	0	0	0	Mo.,N., D.	Mo.	Mo.	Mo, N., D.	Mo.,N., D.
cv. Greyzini F_1	0	0	0	0	0	D.	Mo.	Mo.	Mo., N.	Mo., N.
cv. San Pasquale	0	0	0	0	0	LMo, D, mN.Dw.	L.Mo, D., m.N	LMo., D, mN.	LMo, D, mN, Dw.	LMo, D, mN, Dw.
Cucurbita sp. (ornamental)	0	0	0	0	0	Mo, m. Dw., D.	Mo., m.Dw, D	Mo, m.Dw, D.	Mo, m.Dw., D.	Mo., m.Dw., D.
Cucurbita maxima										
cv. Béjaoui	0	0	0	0	0	Mo, R.L.	Y.S.	YS., s.V.N.	Mo., R.L.	Mo., R.L.
cv. Butternut	Ch.L.	0	Ch.L.	Ch.L.	Ch.L.	I.Ch.RS.	Y., D.,C.L		I.Ch.RS.	I.Ch.RS.

Mo: Mosaic, Ch.L.: Chlorotic lesions, D.: Deformation, Dw.: Dwarfism, S.Mo: severe mosaic, L.Mo: Light mosaic, N.L.: Necrotic lesions, b.N.L.: big necrotic lesions, s.Dw.: Slight dwarfism, m.Dw.: marked dwarfism, V.B.: Vein banding, St.Mo: Streak mosaic, N.: Necrosis, m.N.: marginal necrosis , s.V.N.: secondary vein necrosis , Y.S.: Yellow speckle, R.L: Reduced leaf, Y.: Yellowing, C.L: Cupped leaf, I.Ch.RS: Interveinal chlorosis ringspot, 0: no symptoms.

Table 4. Host reactions to *Prune dwarf virus* infections.

Cucurbit hosts	Local reaction	Systemic reation
Cucumis sativus cv. Marketer	N.r.	s.Mo, Dw.
Cucumis sativus cv. Marketmore 70	N.r.	Mo, s.Dw.
Cucumis sativus cv. Miracross F1	E.N.r.	Mo, s.Dw.
Cucumis sativus cv. Wisconsin SMR 18	C.r.	VB., Dw.
Cucumis sativus cv. White wonder	N.r.	Mo, Dw.
Cucumis sativus cv. Sensation	N.r.	V.Mo, Dw.
Cucumis melo cv.Tendral verde	C. N.r.	Mo, m.Dw.
Cucumis melo cv. Hale's Best	N.r.	Mo, s.Dw., N.
Cucumis melo cv. Planter's Jumbo	N.r.	m.Mo., Dw.
Cucurbita pepo cv. Diamant F1	0	Mo.
Cucurbita pepo cv. Greyzini F1	0	V.C.
Cucurbita pepo cv. San Pasquale	0	s.Dw.
Cucurbita sp. Ornamental	0	Mo., Dw., D.
Cucurbita maxima cv. Béjaoui	0	Y.S., s.V.N.
Cucurbita maxima cv. Butternut	C.r.	Dw.,V.N., D.

N.r.: Necrotic rings, E.N.r.: Extended necrotic rings, C.r.: Chlorotic rings., C.N.r.: Chlorotic and necrotic rings, s.Mo.: Severe mosaic, Dw.: Dwarfism, sDw.: Severe dwarfism, Mo.: Mosaic, V.B.: Vein banding, V.Mo.: Vein mosaic, m.Mo.: Mild mosaic, m.Dw.: Mild dwarfism, N: Necrosis, V.N.: Vein necrosis, D.: Deformation, Y.S.: Yellow speckle, s.V.N.: Secondary vein necrosis, V.N.: Vein necrosis, V.C.: Vein clearing, 0: no symptoms.

necrotic lesions, systemic mosaic and stunting on cucumber (*C. sativus* cv. Miracross F_1) and melon. On squash (*C. pepo*), symptoms were similar but less obvious, while on pumpkin (*C. maxima*) yellow speckling, vein necrosis, stunting and leaf deformation were observed (Table 4).

ELISA

As shown in Table 5, almond was infected with two ilarviruses, PNRSV and PDV. Eight isolates of PNRSV (Peerless, Fournat de Brezenaud, Nec+Ultra, Ferraduel, Ksontini, Mosaic of Sfax, Chlorosis of Sfax and Achak) were found in single-virus infections. Only one pure source of PDV was obtained (Jemmal 1). However, mixed infections occurred, especially in one varietal collection of INRAT at Mornag. No infections by ApMV and ACLSV were detected. Plum was infected with PNRSV and peach with ACLSV.

The antigenic reactivity of 3 PNRSV isolates (Peerless, Khoukhi, Texas) to 9 monoclonal antibodies on natural hosts differed from that on the experimental hosts. With the natural hosts, isolates were divided into two subgroups (Table 6). Peerless and Khoukhi reacted with Mabs 563 and 41, while Texas reacted with all Mabs except 460 and 294. When using experimental hosts, more detailed information was obtained. Virus transmission to the herbaceous hosts yielded a high concentration of virions which made the antigen-antibody reaction more evident, so that each isolate constituted a distinct subgroup. Mabs 460, 563, 503 and 41 had a broad detection range for PNRSV. When typing PNRSV isolates from cucumber, serological variability was correlated with symptom expression. Although Peerless produced the classical mosaic sydrome referred to as line pattern, yellow mottle, vein banding and yellow speckling (Fig. 1a), Khoukhi showed vein clearing, and Texas necrosis of the leaf blade.

PCR detection and differentiation of PNRSV isolates by RFLP analysis of PCR products

The two-step PCR-based assay confirmed that five almond cultivars (Peerless, Fournat de Brezenaud, Ksontini, Nec+Ultra, Ferraduel) grown in two Tunisian mother block stands (GOVPF and GIAF) were infected with PNRSV. A genomic fragment with an expected size of 282 bp was amplified (Fig. 4).

PCR products amplified from all Tunisian isolates were cleaved with the restriction endonucleases *Ava*II and *Nla*III. *Ava*II cleavage of PCR products obtained from Khoukhi and Peerless isolates

Table 5. Reaction of 18 almond, one peach and one plum isolate to four antisera using the ELISA test. The virus isolates were collected from different locations in Tunisia (1991–2000).

Location	Virus isolates	Natural infection				Artificial infection			
		PNRSV	PDV	ApMV	ACLSV	PNRSV	PDV	ApMV	ACLSV
	Almond isolates								
GOVPF (Grombalia)	Nec+Ultra	+	-	-	-	+ (CSM)	-	-	-
	Ferraduel	+	-	-	-	+ (CSM)	-	-	-
	Peerless	+	-	-	-	+ (CSM)	-	-	-
	Ksontini	+	-	-	-	+ (CSM)	-	-	-
	Ferragnès	-	-	-	-	-	-	-	-
	Mazzetto 1	-	-	-	-	-	-	-	-
	Mazzetto 2	-	-	-	-	-	-	-	-
GIAF (Sbikha)	F. de Brezenaud	+	-	-	-	+ (CSM)	-	-	-
	Khoukhi	-	+	-	-	-	-	-	-
	Ksontini	-	-	-	-	-	-	-	-
	Mazzetto	-	+	-	-	-	-	-	-
	Achak	+	-	-	-	+ (CSM)	-	-	-
Jemmal	Jemmal 1	-	+	-	-	-	+	-	-
	Jemmal 2	-	-	-	-	-	-	-	-
Mornag	Mornag	-	+	-	-	+ (CSM)	-	-	-
Sfax	Mosaic of Sfax	+	-	-	-	+ (CMB)	-	-	-
	Chlorosis of Sfax	+	-	-	-	+ (CSM)	-	-	-
	Asymptomatic part of chlorotic tree	-	-	-	-	-	-	-	-
	Peach isolate								
Mehrine	Boutabgaia	-	-	-	+	n.d.	n.d.	n.d.	n.d.
	Plum isolate								
Béni-khalled	Methley	+	-	-	-	n.d.	n.d.	n.d.	n.d.

+, positive; -, negative.
CSM, *Cucumis sativus* cv. Marketer; CMB, *Cucurbita maxima* cv. Béjaoui.
n.d., not determined.

yielded two fragments, of 275 bp and 7 bp (DNASIS, 1991, 1993) indicating the occurrence of one *Ava*II site. Similarly, *Nla*III cleavage of amplified fragments from Texas and Peerless isolates yielded two fragments, of 173 bp and 109 bp (DNASIS, 1991, 1993), also indicating the occurrence of one restriction site. Based on the cleavage patterns of these two endonucleases, the Khoukhi and Texas isolates were distinguishable from one another, but the Peerless isolate could not be distinguished from either.

Discussion

According to field investigations and laboratory work conducted so far in Tunisia, the ilarviruses are the major stone-fruit viruses in orchards as well as in mother block stands. This paper reports on biological (herbaceous and woody plant indicators), serological (ELISA), electron-microscopy, and molecular approaches (RT-PCR) to detect PNRSV, PDV ilarviruses and ACLSV in Tunisia.

Tunisian PNRSV isolates were differentiated by

Table 6. Grouping three *Prunus necrotic ringspot virus* (PNRSV) Tunisian isolates on the basis of their antigenic reactivity to monoclonal antibodies as compared to their infectivity to *Cucumis sativus* cv. Marketer.

PNRSV isolates	Reaction with monoclonal (Mabs) and polyclonal (Pabs) antibodies										Group
	Mabs 460	Mabs 563	Mabs 503	Mabs 242	Mabs 348	Mabs 236	Mabs 41	Mabs 294	Mabs 399	Pabs	
Infectivity to woody natural host (almond)											
Peerless	-	+	-	-	-	-	+	-	-	+	I
Khoukhi	-	+	-	-	-	-	+	-	-	+	I
Texas	-	+	+	+	+	+	+	-	+	+	II
Infectivity to *Cucumis sativus* cv. Marketer											
Peerless	+	+	+	+	-	-	+	+	+	+	I
Khoukhi	+	+	+	-	-	+	+	-	-	+	II
Texas	+	+	+	+	+	+	+	-	+	+	III

+, positive reaction.
-, negative reaction.

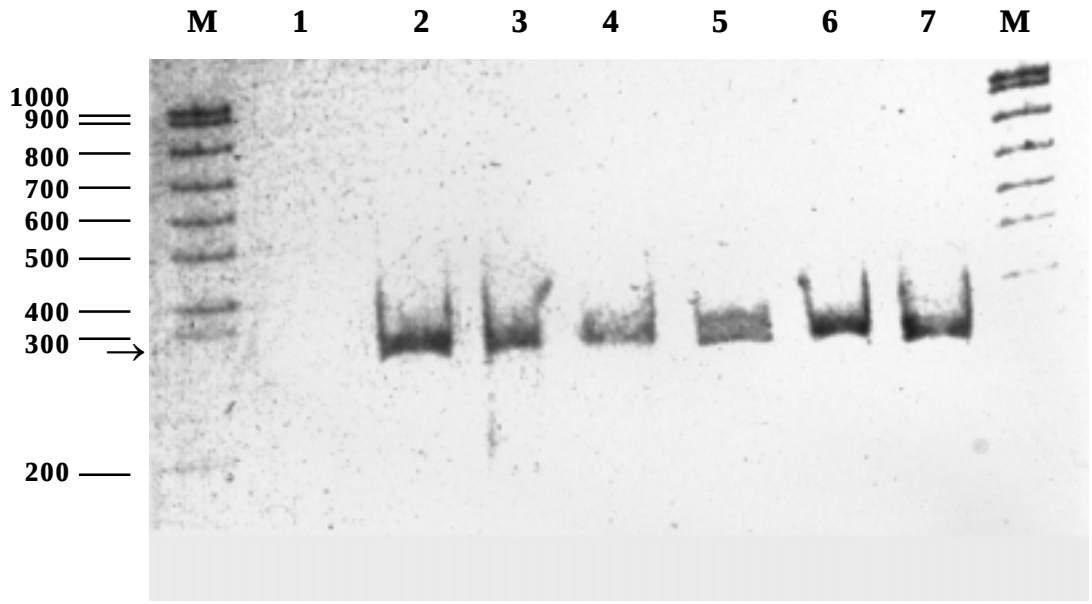

Fig. 4. Electrophoretic analysis of PNRSV PCR products migrated in a 6% polyacrylamide gel (ethidium bromide staining). M, Marker; 1, Healthy control; 2, Infected control; 3, Nec+Ultra; 4, Ksontini; 5, Ferraduel; 6, Fournat de Brezenaud; 7, Peerless. The arrow shows the position of the amplified fragment (282 bp).

bioassays, serotyping with Mabs and RFLP analysis. Biologically, the isolates fell into two groups: 1. Peerless and Ferraduel, and 2. Nec+Ultra, Ksontini and Fournat de Brezenaud. The use of a herbaceous host made it possible to distinguish between PNRSV isolates and revealed for the first time that *Cucurbitaceae* cultivars can be used as PNRSV and PDV indicators.

The use of Mabs for serotyping PNRSV provided a useful and reliable tool for differentiating between three isolates (Peerless, Khoukhi and Texas), placing each in a distinct subgroup. Such differentiation was in good agreement with symptom expression.

RFLP analysis of PCR products also showed that cv. Khoukhi and Texas were different and formed two subgroups. Peerless, however, was not distinguished from either subgroup by this technique. It can be concluded that RFLP was less sensitive in identifying variability than Mabs.

According to Spiegel *et al*. (1999), sequence analysis to differentiate virus strains is laborious and costly, and therefore not the method of choice. The correct interpretation of sequence data also requires extensive preliminary analysis of many different isolates and the identification of strain-specific sequences. At the moment, therefore, serotyping is a more useful and practical technique for typing PNRSV isolates than the molecular methods.

The virus detection and characterization of the PNRSV and PDV isolates reported here are the prelude to a more extensive diagnostic study on virus and virus-like diseases of fruit crops in Tunisia. The current plant health situation of these crops calls for the urgent implementation of a sanitation programme based mainly on the use of virus-free propagating material.

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