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ASSESSMENT OF THE SANITARY STATUS OF POME FRUIT CROPS IN KOSOVO, WITH PARTICULAR EMPHASIS TO VIRUS, VIROID AND BACTERIAL DISEASES

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Abstract

Pome fruits represent very important fruit crops in Kosovo, covering around 50% of the total fruit production. In order to understand the phytosanitary status of pome fruits crops in the Kosovo assessment was carried out for detecting 4 viruses (ACLSV, ASGV, ApMV, ASPV), 3 viroids (ADFVd, ASSVd, PBCVd) and 3 bacteria (*Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *papulans*) on apple and pear. For detection of viruses and viroids serological (ELISA) and molecular techniques (RT-PCR) were used. Concerning bacteria, morphological, biochemical (LOPAT test) and molecular (rep-PCR) tests were performed.
This survey showed that ASPV, ACLSV, ASGV and ApMV were detected in the main apple producing areas in Kosovo, while no pear trees were found infected by these viruses. ADFVd was also detected on apple. Moreover, *Erwinia amylovora* was widely distributed on apple and pear in different cultivated areas.

Key words: Kosovo, pome fruits, viruses, viroids, bacteria

Introduction

In Kosovo there are good climatic and environmental conditions for cultivating pome fruits. Apple is ranks as the first in the total fruit production in the country; pear is grown to a lesser extent while quince groves are very limited to non-commercial level. In order to maximize their outputs with the least possible expenses, growers were constrained to use their own propagating material. This was accompanied by lack of knowledge on diseases and topped by a shortage of an efficient certification program. Therefore, infected material was distributed and introduced into the country and freely circulated.
To assess the presence and spread of most agents of these graft-transmissible diseases in the field, specific detection techniques were brought to light. For viruses detection combining serological (ELISA) and molecular techniques (RT-PCR) were used, whereas for detection and identification bacterias, Gram reaction, morphological and biochemical tests and molecular assays (PCR and rep-PCR) were performed. .

Material and Methods

Several visual inspections and field observations were carried out during spring and summer time 2012 for this survey. The sampling from 303 apple and 41 pear trees was performed across the Kosovo territory, covering the main pome fruit producing areas. For the viruses and viroids detection 244 and 60 samples respectively were chosen, while 100 samples were assayed for the bacteria detection. A total of 244 samples were collected, from trees grown in mother plots and commercial orchards located in Prishtina, Lypjan, Mitrovica, Ferizaj, Klina, Burim and Peja municipalities (Fig. 1). Inspected trees varied in age from two to seventeen years old and almost all are imported cultivars. From each of the 219 apple and 25 pear trees three twigs 15 cm in length with leaves were cut, labelled and stored at 4°C for about 3-4 days and then used for further laboratory assays. Visual inspections for specific symptoms of virus, viroid and bacterial infections were also carried out during field surveys. For the bacteria surveys, samples were collected from apple and pear trees grown in mother plots and commercial orchards in Prishtina, Kamenica, Peja, Mitrovica and Ferizaj areas. In Peja and Ferizaj areas, almost all collected samples, showed symptoms which could be associated to the bacterium *Erwinia amylovora* infection.

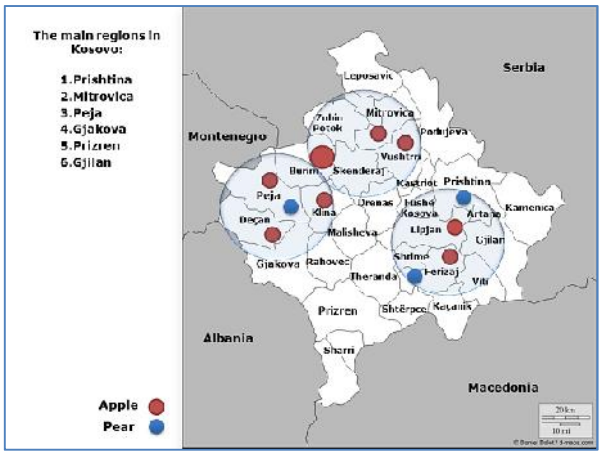

Figure 1. Map of inspected areas related with virus, viroid and bacteria surveys

All investigated samples were tested by DAS-ELISA (Clark and Adams, 1977) for detection of ACLSV, ApMV and ASGV, using commercial kits from Bioreba (Switzerland). Moreover, a total of 60 samples were selected to perform the detection of ASPV virus and viroids. Total RNA was extracted from leaves of pome fruit trees using the silica RNA extraction method (Foissac *et al*., 2001). Relatively to the detection of Apple stem pitting virus (ASPV) (Menzel *et al*., 2002) and three viroids (ASSVd, ADFVd and PBCVd) by RT-PCR, according to Di Serio *et al*. (2002) and Lolic *et al*. (2007), cDNA synthesis was performed using 5 µl of viral template, mixed with 1µl of random hexamers primers (Boehringer Mannheim, GbmH) (0.5 µg/µl), denatured at 95^{o}C for 5 min then immediately cooled in ice.

A 2 hours reverse transcription reaction was carried out at 37^{o}C after adding 4µl M-MLV (Moloney-Murine Leukemia Virus) buffer 5X (50 mMTris-HCl pH 8.3, 75 mMKCl, 3 mM MgCl2), 2 µl of 10 mM DTT, 0.5 µl of 10 mMdNTPs, and 200 units M-MLV reverse transcriptase (Invitrogen Laboratories, USA). Inactivation of M-MLV RT was obtained by incubation at 72°C for 7 min.PCR was carried out in a final volume of 25 µl using 2 µl of cDNA, 1 µl of corresponding reverse and forward primers each, 5 µl of GoTaq buffer (5x), 1 µl of dNTPs (10mM each), 1.5 µl MgCl2 (50 mM). The obtained mix was subjected to thermal cycling at 95^{o}C for 5 min followed by 35 cycles at 94^{o}C for 35 sec, 55^{o}C for 45 sec, and 72^{o}C for 50 sec. The final extension step was at 72^{o}C for 7 min. PCR products in agarose gel 2% were visualized under the UV light.

Relatively to the viroid detection, reverse transcription was performed with cDNA reverse transcription kits according to the manufacturer’s recommendations (Applied Biosystems, Foster City USA) and using the total RNA preparations extracted by the method of Foissac *et al*. (2001). The cDNA obtained after reverse transcription was amplified with GoTaq DNA polymerase and the

appropriate primers pair and annealing temperatures. PCR cycling conditions were 95^{o}C for 5 min followed by 30 cycles of denaturation at 94^{o}C for 40 sec, annealing at the appropriate temperature for 40 sec, and extension at 72^{o}C for 50 sec. The final extension step was at 72^{o}C for 7 min. PCR products were separated by electrophoresis in 2% agarose gel and stained with ethidium bromide.

Concerning the bacteria detection, during the winter and spring period, 2012 the asymptomatic samples (bud sticks) were collected, sunken leaf discs in PBS, were taken and put in Petri dishes containing NA and KB agar media. However, during the June, 2012, the symptomatic bud sticks and leaves, were macerated in few drop of SDW in a sterile eppendorf tube using sterile scalpel and forceps, thirty minutes after the maceration, 100µl of macerated tissues were taken from and streaked onto NA and KB agar media using sterile loop. The plates were then incubated at 27^{o}C ± 1^{o}C for 1 week and observed daily for bacterial growth (Jones and Geider, 2001; King, *et al*, 1954). Suspected colonies of *Erwinia amylovora* and *Pseudomonas* spp. were purified and incubated at 27^{o}C ± 1°C. The Kosovan isolates were compared with a collection strains from different countries and hosts, through biochemical tests (Table 1).

Table 1. Reference strains of *E. amylovora* and out group strains used for differentiation assays

Strain	Scientific name	Host	Isolation date	Source
Ea I - 204	*Erwinia amylovora*	*Pyruscommunis*	2002	Italy
Ea I - 208	*Erwinia amylovora*	*Pyruscommunis*	2002	Italy
Ea DZ – 11	*Erwinia amylovora*	*Pyruscommunis*	2010	Algeria
CFBP – 1754	*Pseudomonas syringae*pv.*papulans*^{T}	*Malussylvestris*	1973	Canada
CFBP – 311	*Pseudomonas syringae*pv. *syringae*	*Pyruscommunis*	1962	France
CFBP-5472	*Pseudomonas syringae*pv. *syringae*	*Malusdomestica*	1988	Canada
CFBP-1670	*Pseudomonas savastanoi* pv. *savastanoi* ^{T}	*Oleaeuropea*	-	Ex-Yugoslavia
LMG – 2408	*Pectobacterium carotovorum* subsp. *carotovorum*	*Zantedeschiaaethiopica*	1950	UK
CFBP - 1346	*Dickeya chrysanthemi* biov. *chrysanthemi*	*Chrusamthemummaximus*	1969	Italy

*EaI 204, and EaI 208 were provided by M. Scortichini (CRA, Centro di Ricerca per la Frutticoltura, Rome, Italy), *EaDZ (Laala*et al.*, 2012); *LMG, LaboratoriumvoorMicrobiologieUniversiteit Gent, Belgium;
*CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France.

All colonies obtained were subjected to the LOPAT test (Schaad *et al*., 2001) for *Pseudomonas* spp. and to the EPPO key tests for the *E. amylovora* identification. Concerning the molecular tests, DNA extraction was performed by using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich-USA), following the manufacturer's recommendations.
According to Versalovic *et al*. (1991; 1994) and Louws (1996) PCR reactions were carried out in a 25 µl volume for each reaction; the protocol using BOX, ERIC and REP primers was used for the PCR assays. The eletcrophoretical profile of the agarose gel obtained by rep-PCR using primers sets, respectively, was compared with the reference strains (Table 1).

Results and Discussion

Twenty four out of 244 samples tested by DAS-ELISA, confirmed to be infected by at least one virus, 12 reacted positively to ACLSV, 11 were positive to ASGV and only 1 sample was reacting positively to ApMV.

The positive ACLSV trees were recorded in municipalities of Peja, Burim, Klina and Ferizaj, while the ASGV was detected in the municipalities of Peja, Klina and Burim. Peja municipality was the most infected area (Fig. 2).

Among the different cultivars tested, the total ASGV infection was higher on Idared (46%), followed by Red Delicious (36%), whereas the Jonagold and some undetermined apple cultivars presented the same infection rate (9%). Moreover, ApMV was detected only in one Idared cultivar sampled in Peja area.

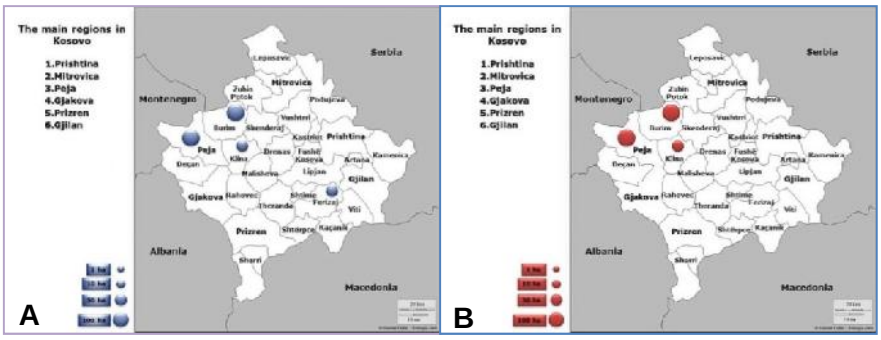

Figure 2. Distribution of ACLSV (A) and ASGV (B) in Kosovo

ASPV was detected in the municipalities Mitrovica, Ferizaj, Klina, Burim and Peja. Burim municipality was the most infected area, followed by Klina, Ferizaj, Mitrovica and Peja (Fig. 3). The highest ASPV infection was found on Granny Smith cultivar (17%) followed by Golden Delicious and Jonagold (14 %), while the lower infection was found on newly introduced cultivars Gloster, Starting and Braeburn (3% each of them).

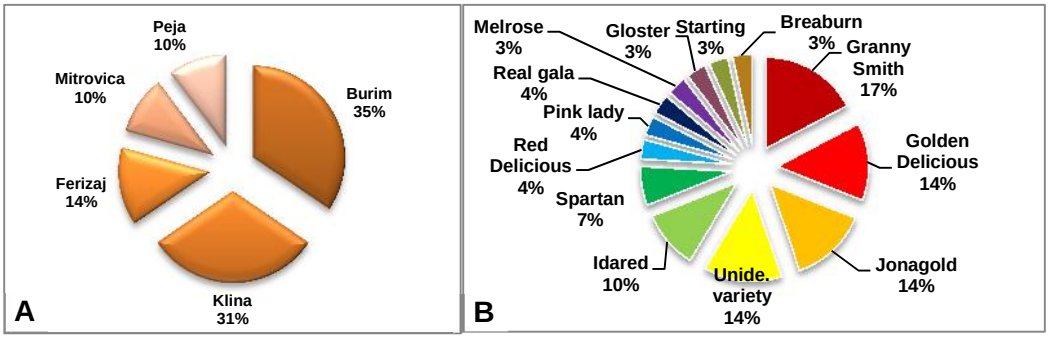

Figure 3. Incidence and localisation (A) and distribution of ASPV infected trees among cultivar (B)

The present study shows that ACLSV, ASGV, ApMV and ASPV are widely distributed in commercial orchards where the propagative plant material was imported by some farmers from Serbia (47%), while lower infection rate (6%) was detected on trees which were planted with plant propagative material originated from Albania.

The detection of the viruses was obtained in orchards of different ages however, in general the presence of the viruses was higher (47%) in the older ones (more than 12 years old), and higher was also the infection rate of young infected trees (35%) ()up to 5 years old orchards.

The presence of the viruses was similar in the older trees (47%) and the infected young trees (35%). Moreover, bacteria were isolated from symptomless samples collected in Prishtina, Kamenica and Mitrovica, whereas, in isolated bacteria from Peja and Ferizaj areas almost all the trees were showing symptoms which are associated to the Fire blight disease.

After 3 days, the observed colonies in NAS medium were 4-5 mm in diameter, circular, convex and did not show any yellow colour which can be characteristic of *Pantoea* isolates (Janda and Abbott, 2006). Meanwhile, other colonies observed in CCT plates at 27^{o}C were 3-4 mm in diameter, mucoid with shiny surface, semi-transparent and slight violet, characteristically of *E. amylovora.* Relatively to the biochemical characteristics, among the 70 tested isolated, 24 reacted positively to the Levan and the hypersensitivity test on tobacco. The last were subjected to rep-PCR assays, using REP, BOX and ERIC primers, interestingly, 21isolates showed the pattern which was similar to the reference strains of *E. amylovora* (Table 1). While only one obtained profile was similar to the *P. syringae* pv. *syringae* reference.

Conclusions

Through a wide survey on pome fruit trees in Kosovo, useful information was obtained on the phytosanitary status of these crops in the country. Most of the sampled trees showed to be infected by at least one of the four viruses ASPV, ACLSV, ASGV and ApMV on apple. No viral infection was revealed from pear. This study evidenced for the first time the presence and real distribution of ACLSV, ASPV, ApMV and ASPV infections in the country. The ASPV showed to be the most prevalent virus in the country with an average rate of 48%, followed by ACLSV and ASGV (5%) and ApMV (1%) respectively. Moreover, this study allowed detecting for the first time on apple, the Apple dimple fruit viroid (ADFVd) reaching an infection rate of 18%. However, no virus and viroid were detected in the Eastern part of Kosovo. Furthermore, *Erwinia amylovora* was extensively distributed on apple and pear in several localities of Kosovo, confirming the wide distribution of this bacteria in the Balkan area. On the other hand, *Pseudomonas syringae* pv. *Syringae* which was detected molecularly in a single apple tree, constitute the first report of this bacteria in the country. Older cultivars(Idared, Golden Delicious and Red Delicious) showed to be the most infected cultivars comparing to the newly introduced cultivars in Kosovo. The viral and bacterial infections seem to occur in most of the pomefruit growing areas. In addition, viroids were also found in cultivated orchards.

This work will constitute a basic study on the prevalence of pome fruit viral and bacterial diseases in Kosovo and will allow the national authorities to establish a national program to improve the quality of propagative material and the fruit production.

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