SEED AND BUD PROPAGATED PLANTLETS CAN BE USED TO DOUBLE CHECK THE PRESENCE OF MIXED VIRAL INFECTIONS AT APPLE

SUMMARY
Two apple cultivars, Golden delicious and Starking grown in Devoll, Albania, were analyzed to verify the rate of infection by ApMV (Apple Mosaic Virus), ASGV (Apple Stem Growing Virus), ACLSV (Apple Chlorotic Stem Virus) and ASPV (Apple Stem Pitting Virus) using in vitro propagated plantlets, and results taken from different categories of explants and among collections were compared based in the fact that template concentrations were equal. Samples, in total 450 trees, were buds taken from branches collected in early spring from symptomless trees, and seeds taken from fruits at three collection orchards during consecutive years 2013, 2014, 2015. Total RNA was extracted from plantlets grown in vitro from buds and seeds, which were used as template for One-step RT-PCR detection of the viruses. Results showed that viruses are present in both cultivars from the three collection orchards. Concentrations of amplicons varied among cultivars, explants categories and from collection to collection, which suggested different infection rates. The presence of mixed latent viral infection was discussed in terms of a possible mechanism of cross-protection among viral strains already amplified, and concentration of amplicons considered proportionate to the infection rate, was seen as a tool to monitor the situation in collections even in the absence of field symptoms.

Keywords: ApMV, ASGV, ACLSV and ASPV, RT-PCR, apple cultivars, viral phytopathology

INTRODUCTION
Apple is susceptible to infection by pathogens, especially viruses such as ApMV (Apple Mosaic Virus), ASGV (Apple Stem Growing Virus), ACLSV (Apple Chlorotic Stem Virus) and ASPV (Apple Stem Pitting Virus). These viruses usually do not induce visible disease symptoms in the infected trees and fruits, although the infection leads to reduction in fruit yield and quality. Myrta A., (1998) reported that apple fruit production in Albania ranked second among the horticultural crops after grapevine, and bypassed that of olive and citrus. Since that time, the international or the native cultivars produced in Albania still lack the quality demanded by the market, and vegetatively propagated materials

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circulate without restrictions, which makes the determination of incidence and
distribution of viral diseases important. A preliminary account of the presence of
viruses of pome fruits in Albania was conducted in 2004, and results based on
biological indicators and serological methods showed that rate of infection was
100% in apple with prevailing viruses ASPV, ACLSV, and ASGV (Myrta et al.,
was found in Albania in apple only, while Grazhdani et al., 2014 described RT-
PCR based data on ApMV infection in apple from Devolli area. In this context,
from Albania were reported a number of research works, which address the
methodology for sanitation of pome and stone fruit-trees (Sota & Kongjika, 2014
a,b,c). Generally apical meristems without having leaf primordial, have the
highest probability of producing plantlets that are virus-free, however they do
have the lowest probability of surviving in culture medium. According to Xhixha
et al., 2014; Grazhdani et al., 2014, several protocols were established to avoid
polyphenolic oxidation of explants of some cultivars of Malus sps. during early
micropropagation stages. On the other hand, the majority of plants are raised
through true seeds. According to Sastry (2013) more than 231 viruses and viroids
are seed transmitted. Susuri and Myrta (2012) reported that the four above
mentioned viruses are prevalent at apples cultivated in Albania, that they are
distributed mainly vegetatively, and that there is no evidence on seed
transmission of the diseases. Considering the knowledge on actual status of viral
disease at apple in Albania, and in accordance to EPPO, 1999, these plants have
to be tested. Nowadays the basic methods for the detection are ELISA and RT-
PCR (Posnette, et al, 1963; Hassan et al., 2005; Menzel et al., 2002; Kinard et
al., 1996; Watpade et al., 2013, etc). According to Saade et al., 2000, nonisotopic
molecular hybridization and multiplex RT-PCR methodologies were developed
that could detect all these viruses simultaneously. Meanwhile, the presence of a
number of mixed viral infections at apple, already reported in the Mediterranean
(Hassan et al, 2005; Di Terlizzi et al., 2003, etc) raises the need for further
investigations on the issue. This study aimed to detect viruses at in vitro
propagated plant material based on RT-PCR methodology, provide
considerations on the efficacy of in vitro propagation of apple from seeds and
buds as a tool for phytosanitation, and offer an indication on possible
transmission of the infections from seeds.

MATERIAL AND METHODS

Samples to be analyzed: In vitro propagated plant material from cultivars
Golden delicious and Starking, originated from seeds and buds of trees grown in
three collection orchards (Hocisht, Bitincke and Cangonj) was used to extract
total RNA. For each category were sampled 50 individual plants, and RNA mixes
were prepared (Table 1).

Total ARN extraction and RT-PCR: 120mg of leaf tissue were macerated
with mortar and pestle in a cooled mortar in the presence of 600µl 1X extraction
buffer (1% lauryl sarcosine, 2% SDS, 0,1Mglycine-NaOH, pH9) and mixed with
Seed and bud propagated plantlets can be used to double check the presence of viral... 

Table 1. Sample numbering, composition, origin and collection from where plant material was taken in the area of Devoll, Albania.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Cultivar</th>
<th>Plantlets origine</th>
<th>Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Starking</td>
<td>Seeds</td>
<td>Hocisht</td>
</tr>
<tr>
<td>2</td>
<td>Starking</td>
<td>Seeds</td>
<td>Cangonj</td>
</tr>
<tr>
<td>3</td>
<td>Golden delicious</td>
<td>Seeds</td>
<td>Hocisht</td>
</tr>
<tr>
<td>4</td>
<td>Golden delicious</td>
<td>Seeds</td>
<td>Cangonj</td>
</tr>
<tr>
<td>5</td>
<td>Golden delicious</td>
<td>Buds</td>
<td>Hocisht</td>
</tr>
<tr>
<td>6</td>
<td>Golden delicious</td>
<td>Buds</td>
<td>Cangonj</td>
</tr>
<tr>
<td>7</td>
<td>Golden delicious</td>
<td>Buds</td>
<td>Bitincke</td>
</tr>
<tr>
<td>8</td>
<td>Starking</td>
<td>Buds</td>
<td>Bitincke</td>
</tr>
<tr>
<td>9</td>
<td>Starking</td>
<td>Buds</td>
<td>Cangonj</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total RNA was used as template for One-step RT-PCR performed according to HS-RT-PCR Kit of SIGMA, using virus specific primers for ASPV, ASGV, ACLSV and ApMV, and cycling conditions according to W. Menzel et al., 2002. Amplicons were verified in 1.5% agarose gels in TAE along with 100bp molecular marker, and photos were taken under UV ligh.

RESULTS AND DISCUSSION

RT-PCR reaction using specific primers for ApMV gave an amplicon of expected size for all the sampled material of in vitro categories. The concentration of amplicons from cultivar Starking (samples no. 1, 2) was higher compared to cultivar Golden delicious (samples no. 3, 4, 5, 6).

![Figure 1](image)

Figure 1. Amplicons of ApMV gene fragment originated from in vitro propagated plantlets from seeds of cultivar Starking and Gold. From right to left: Molecular marker 100bp, samples 1-6; from all of them is amplified a fragment of expected size 450bp.

Samples no. 6, 7, 8, 9 (Fig. 2), which present in vitro plant material originated from buds are also infected by ApMV, and the dimensions of amplicons are of two very close categories (no. 7-8 are slightly bigger than no. 6-9). Comparing the amplicon concentrations our data showed that samples originated from seeds from cultivar Starking/Cangonj (no. 2) are more concentrated than those originated from buds (no. 9); vice-versa for cultivar
Golden delicious (Fig.1) sample from buds (no.5) is more concentrated than the one from seeds (no.3).

The amplification of ACLSV gene fragment (Fig. 3 and 4) was not achieved from samples no.2 and no.6, while corresponding (from the same mother plant) plants grown from buds (no.9) and seeds (no.4) were contaminated. These results serve to double check the sanitary status, and understand that mother plants are contaminated.

Figure 5 shows that all samples (except sample 9 and control) are contaminated from ASPV. Samples no. 3, 4 and 6, (cultivar Golden delicious) have an amplicon of low concentration in comparison with rest of samples, which indicate a lower level of infection of mother plants from which were taken.

The amplification of ASGV (Fig. 6) showed that samples originated from seeds (no. 3, 4) are not infected, while those originated from buds (no. 5, 6) of the same cultivars from the same collections are infected; Samples no. 7, 8 present plants of two cultivars grown from buds of the same collection orchard, are infected from the four viruses and the amplicon concentration is almost the same in all reactions; As Fig.6 shows, plants from collection Cangoj (no. 2, 4, 6, 9) are of three categories of sanitary status: not infected (no. 4, 9 did not produce viral amplicon), slightly infected no. 6 (the amplicon concentration is low), and infected (no2. gave an amplicon with high concentration). Plants from collection of Hocisht (no. 1, 3, 5) are infected (no.3 has a minor product); Plants from collection of Bitincke are infected (no. 7, 8).
Seed and bud propagated plantlets can be used to double check the presence...

Nowadays, are reported a number of diagnostic field tests for different viruses of fruit trees, however, the situation gets complicated because the field symptomatology can be similar for ApMV, ASGV, ASPV, ACLSV (Susuri & Myrta, 2012). The use of woody indicators has disadvantages as being time consuming, expensive and the results may be difficult to interpret. ELISA tests, even though used widely, often fail because of low virus titer and the inhibitory effects of compounds in the sap of woody plants (W. Menzel et al., 2002). Therefore, PCR techniques provide a fast, reliable and cheaper alternative. As described by Fig.7 samples from collections of Hocisht, Bitincke and Cangonj are infected by mixed viruses (1-4 viruses), and those from Cangonj are less infected.

Fig.8 informs on the rate of infections at each sample. It results that in a pool of 100 plants from the collection of Hocisht are found present 2,6 out of 4 types of viruses; At the collection of Cangonj are found 1,5 out of 4 types of viruses, and at the collection of Bitincke are found 4 out of 4 viral categories. It is known that if a plant is already infected with one virus strain, it resists infection by different strains of the same virus. Posnette and Cropley (1952, 1956) showed that mild strains of ApMV would protect trees of different apple cultivars against the effects of severe strains, and Thomsen (1975) concluded that symptom development depended on the apple cultivar and the virus strain, and varied from year to year and among trees of the same cultivar. In Albania, the RT-PCR amplification of different isolates of viruses (Bacu et al., 2014) was described for apples of three cultivars from Kukes, which is an area of important production in the country. As for the viral fragments amplified from cultivars Golden delicious and Starking of the area of Devoll, work will continue to verify if they are single or comigratory products.
Figure 7. Comparison of the presence of mixed viral infections at collections of Hocisht, Cangonj, and Bitincke.

Figure 8. Description of the rate of infection of each sample of the three collections.

Apple virus diseases are mainly spread thorough vegetative propagation, however, possible transmission patterns are mechanical, grafting, seeds, pollen, nematodes and insects. Among the last, seed transmission depends on the ability of a virus to invade and replicate in the reproductive tissues of its host, and to overcome without damage the physiological modifications associated to seed maturation (Bassi D. & Martelli G.P., 2003). According to previous reports (Susuri & Myrta, 2012) there is no evidence on seed transmission of the four diseases in Albania. Our data show that plantlets generated from seeds (samples no.1, 2, 3, 4) were infected from ApMV (100%), from ACLSV (75%), from ASPV and ASGV (50%), while plants generated from buds (samples no.5, 6, 7, 8, 9) showed a higher rate of infection (100% from ApMV; and 80% from the rest of the viruses). Plantlets generated from seeds of cultivar Golden delicious were not infected by ASPV and ASGV (samples no. 3, 4), while those generated by buds (from the same mother plants) were infected (samples no. 5, 6). Because plant material analyzed in this study were 450 individuals (200 of which generated from seeds; see Table 1), work will continue for the evaluation of possible transmission of the four viral diseases thorough seeds in a bigger population.
CONCLUSIONS

Objective of the study was the control of sanitary status of apple plantlets of cultivars Golden delicious and Starking, propagated from seeds and buds of mother plants grown in three collection orchards in the area of Devoll, Albania.

Results prove that in total 68% of plantlets propagated by seeds and 85% of plantlets propagated thorough buds were infected by mixed viral infections of ApMV, ASGV, ACLSV and ASPV.

In few cases plantlets (originated from the same mother plant) either from seeds or buds failed to give amplicons. Thus, we conclude that the use of two categories of explants serves to double check the sanitary status of mother plants.

The rate of mixed infections at three collections, measured as the number of categories of viral infections per 100 plants, shows that mixed infections (ApMV, ASGV, ACLSV and ASPV) are more prevalent in Bitincke, Hocisht, and less in Cangonj.

The plantlets originated from the collection orchard of Bitincka in most of the cases gave amplicons of higher concentration than the ones of collection orchards of Cangonj and Hocisht.

Considering that reactions conditions and template concentrations were equal, we believe that the concentration of amplicons is proportionate to the level of infection, and can be used to monitor the sanitary situation in collections even in the absence or unclear field symptoms.

REFERENCES


