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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*., 2004). According to Susuri & Myrta, 2012, the Apple Mosaic Virus (ApMV) 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

an equal volume of water-saturated acidic phenol. After a second extraction with phenol-chloroform, total nucleic acids in the final aqueous phase were precipitated with ethanol and resuspended in 50µl of RNA-se free water.

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

Sample No.	Cultivar	Plantlets origine	Collection
1	*Starking*	Seeds	Hocisht
2	*Starking*	Seeds	Cangonj
3	*Golden delicious*	Seeds	Hocisht
4	*Golden delicious*	Seeds	Cangonj
5	*Golden delicious*	Buds	Hocisht
6	*Golden delicious*	Buds	Cangonj
7	*Golden delicious*	Buds	Bitincke
8	Starking	Buds	Bitincke
9	Starking	Buds	Cangonj
10	Control		

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).

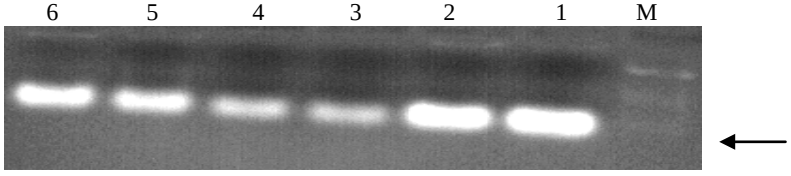

Figure 1. Amplicons of ApMV gene fragment originated from *in vitro* propagated plantlets from seeds of cultivar *Starking* and *Gold*. From right to left: Molecular marker 100bp, samples 1-6; from all of them is amplified a fragment of expected size 450bp.

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).

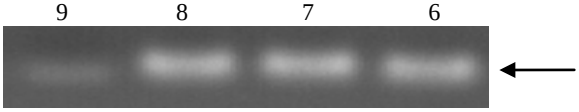

Figure 2. Amplicons of ApMV gene fragment. From right to left: samples 6-9; sample; from all of them is amplified a fragment of expected size ~ 450 bp.

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.

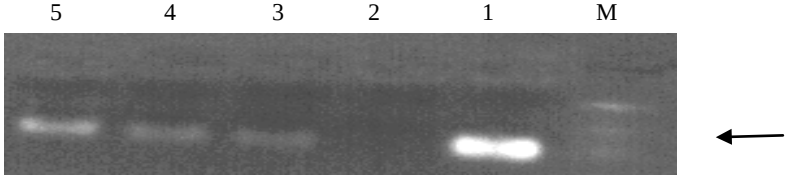

Figure 3. Amplicons of ACLSV gene fragment. From right to left: Molecular marker 1kbp, samples 1-5; from samples 1,3,4,5 is amplified the fragment of expected size 670 bp.

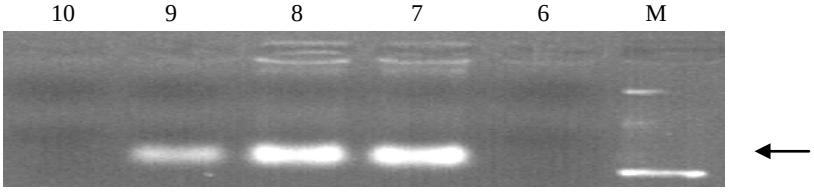

Figure 4. Amplicons of ACLSV gene fragment. From right to left: Molecular marker 1kbp, samples 6-9; sample 10 is control; from samples 7, 8, 9 is amplified the fragment of expected size 670 bp.

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 Cangonj (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).

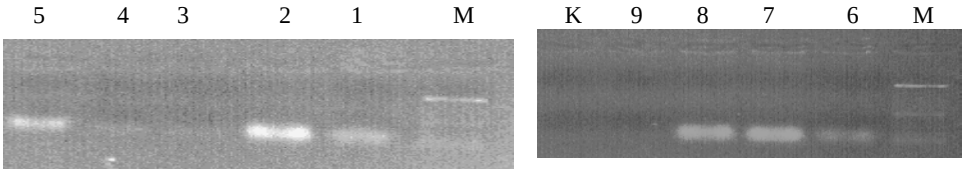

Figure 5. Amplicons of ASPV gene fragment. From right to left: Molecular marker 1kbp, samples 6-9; sample 10 is control; Molecular marker 1kbp- samples 1-5 from which the amplicon of expected size 370bp is produced from 1, 2, 3, 4, 5, 6, 7, 8.

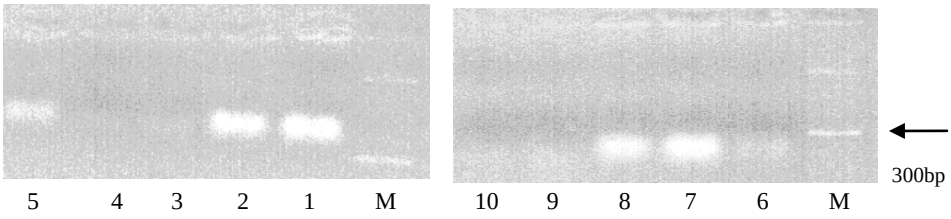

Figure 6. Amplicons of ASGV gene fragment. From right to left: Molecular marker 1kbp, samples 6-9; sample 10 is control; Molecular marker 1kbp- samples 1-5from which the amplicon of expected size 273 bp is produced from samples 1, 2, 5, 6, 7, 8.

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.

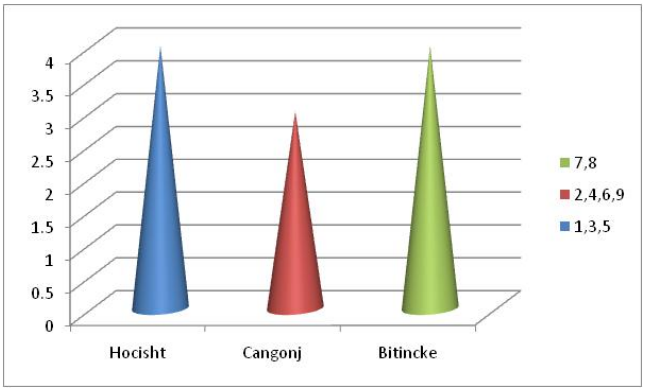

Figure 7. Comparison of the presence of mixed viral infections at collections of Hocisht, Cangonj, and Bitincke.

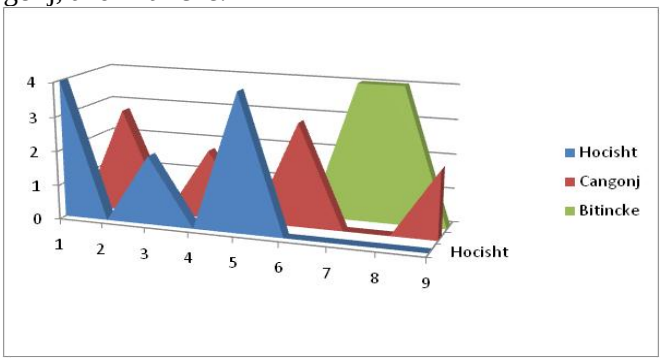

Figure 8. Description of the rate of infection of each sample of the three collections.

Apple virus diseases are mainly spread thorough vegetative propagation, however, possible transmittion 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 transmittion 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.

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