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Original article

Diagnosis of Peach latent mosaic viroid (PLMVd) and Hop stunt viroid (HSVd) by RT-PCR using different extraction protocols

Peach latent mosaic viroid (PLMVd) ve Hop stunt viroid (HSVd)'in farklı ekstraksiyon metotları kullanılarak RT-PCR ile teşhisi

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ABSTRACT

RT-PCR method was performed using four different nucleic acid extraction methods to identify viroids; peach latent mosaic viroid (PLMVd) and hop stunt viroid (HSVd) which causes serious damage to stone fruits. Leaf samples were collected from fruit orchards showing viroid-like symptoms in İzmir province. Silica capture, citric buffer, lithium chloride, and ames buffer methods were used to extract total nucleic acids. The four extraction methods were compared using samples collected during the vegetation period from naturally infected trees (plum, apricot, and peach). They were evaluated with RT-PCR tests. In 64 stone fruit tree samples, only the silica capture method gave reliable and accurate results in RT-PCR molecular tests for the detection of PLMVd and HSVd. The other wielded nucleic acid extraction methods were found to be ineffective for the isolation of the viroid RNAs.

INTRODUCTION

Stone fruits are significant in the agricultural economy and contain 30% of fruit production; in total 3 million tons of stone fruits are produced in Türkiye (TÜİK 2019). Plant pathogenic viroids are small, single-stranded RNA molecules that infect plants and cause disease (Góra-Sochacka 2004). Viroids do not code or change proteins. They show their effects in host plants with plant enzymes. They cause alteration of the genes that regulate plants' growth and development. In previous studies, it was determined that viroids cause disease not only in highly structured plants but also induce disorders in hosts from a lot of various families and genera (Góra-Sochacka 2004). Potato, tomato, hop, coconut, grapevine, citrus, avocado, peach, apple, pear, and chrysanthemum are among the hosts that viroids cause disease. Therefore, the wide host range of viroids facilitates their spread and transmission. Viroids are transmitted by grafting, Cuscuta spp., vector insects, seed, pollen, and mechanical ways (Hataya et al. 2017).

They systematically consist of two families. These families are; Pospiviroidae and Asunviroidae. In viroid diseases, it is crucial to identify the viroid with all aspects, determine its strain and hosts, and finally detect the viroid with the rapid diagnostic methods for the control (Flores et al. 2004). They cannot be detected by the serological methods because of the absence of their protein coats but can be detected by

molecular methods such as hybridization and RT-PCR in the diagnosis of plant viroid diseases (Hataya et al. 2017, Ragozzino et al. 2004). It is often necessary to extract the nucleic acids from plant tissues to detect and study these viroids. There are many different nucleic acid extraction protocols available, and the choice of method depends on several factors such as the nature of the sample, the downstream application, and the sensitivity of the detection method.

Diagnosis and detection are as considerable as cultural and sanitation practices in preventing the spread of viroid diseases. Indicator plants, molecular hybridization, and Reverse Transcription Polymerase Chain Reaction (RT-PCR) methods are reliable and the most used among the detection methods.

RT-PCR has been widely used for a long time in molecular biology studies. Extraction and purification methods are also very important in obtaining the desired amount of virus and viroid RNAs from plants (Akbas and Degirmenci 2010, Schepetiuk et al. 1997, Wiedbrauk et al. 1995). There are many different nucleic acid extraction protocols available, and the choice of method depends on several factors such as the nature of the sample, the downstream application, and the sensitivity of the detection method. Many materials existing in the structure of the plant as cytosol, gum, and phenolic compounds play a preventative role in the reaction of RT-PCR (Martelli et al. 1994). The main challenge in RT-PCR application occurs in the process of the preparation of nucleic acid extraction in good quality. This problem is encountered generally in studies conducted on woody plants, especially with the origin of Malus and Prunus (Korschineck et al. 1991). Mostly used nucleic acid extraction procedures cannot terminate the connection with polysaccharides and phenolic compounds preventing RT-PCR (Demeke and Adams 1992).

Herein, it was aimed to determine the best RNA extraction method for agents representing both viroid families causing diseases in orchards in Türkiye and the world. For this, peach latent mosaic viroid (PLMVd) belonging to the family Pospiviroidae and hop stunt viroid (HSVd) belonging to the family Asunviroidae, which are the most significant viroids that cause diseases wherever stone fruits are grown, were selected.

MATERIALS AND METHODS

PLMVd and HSVd isolates from the previous studies and the 64 leave samples collected from apricot, peach, and plum trees, positive and negative controls consist of the main materials of the study. These isolates were used in RT-PCR tests..

Field surveys and sampling

Plant leaves showing viroid-like symptoms were collected from various fruit orchards in İzmir province. Sampling was conducted during the spring and summer months. Collected leaf samples were put into the polyethylene bags, marked with a code, and kept in an icebox during transportation to the laboratory. All samples were kept at -20 °C in deep freeze until nucleic acid extraction.

Total nucleic acid (TNA) extraction

Total nucleic acid extraction was carried out using four different methods. These methods are; silica capture (Foissac et al. 2000), citric buffer (Wetzel et al. 1992), lithium chloride (Hughes and Galau 1988), and ames/chloroform buffer methods (Laulhere and Rozier 1976, Podleckis et al. 1993).

RT-PCR (Reverse transcriptase polymerase chain reaction) method

In the PCR reaction, complementary DNA (cDNA) was synthesized for the total nucleic acid of viroids extracted from plants. For this purpose, protocols of the cDNA (Super Script RNAase H) kit supplied by Invitrogen (Invitrogen, TECH-LINESM U.S.A.) company were followed. The PCR process was handled at 50 µl volume according to the procedure advised by Fermantas Company. Primers used in the RT-PCR test were given in Table 1.

25 µl 2x PCR master mix. 1 µl primer 1, 1 µl primer 2, 2 µl cDNA, and 21 µl nuclease-free water were added to the sterile PCR tubes. Amplifications were carried out in an analytikjena thermal cycler in the cycling conditions, given in Table 2 for each viroid (Candresse et al. 1995). PCR products were separated by electrophoresis in 1.5% agarose gel in TAE buffer at 105 V for 60 minutes and stained with ethidium bromide and visualized under UV light.

Table 1. Viroid primers used in RT-PCR analysis

Viroid	Primer	Base Pair	Sequence	Position	Reproduced DNA (bp)	References
PLMVd	cPLMVd	25	5'-AACTGCAGTGCTCCGAATAGGGCAC-3'	91-115	337	Loreti et al. (1999)
	hPLMVd	25	5'-CCCGATAGAAAGGCTAAGCACCTCG-3'	116-140		
HSVd	VP19	26	5'-GCCCCGGGGCTCCTTTCTCAGGTAAG-3'	60-85	300	Astruc et al. (1996)
	VP20	25	5'-CCCGGGGCAACTCTTCTCAGAATCC-3'	80-102		

PLMVd, peach latent mosaic viroid; HSVd, hop stunt viroid.

Table 2. Followed programs in RT-PCR analysis

PLMVd	30 cycles 95 °C 3 min	30 cycles 95 °C – 30 s 60 °C – 45 s 72 °C - 45 s	30 cycles 72 °C 7 min
HSVd	1 cycle 95 °C 3 min	30 cycles 95 °C – 30 s 60 °C – 45 s 72 °C - 45 s	1 cycle 72 °C 7 min

RESULTS AND DISCUSSION

A total of 64 samples, 2 positive control, and 2 negative control plants were employed in the study. For the four nucleic acid extraction methods; two different primer pairs were put into account. Both primer pairs were worked with the silica capture method in previous assays and their existence was validated. PLMVd was found in 12 out of 64 tested samples, and hop stunt viroid was found in 8 samples by using the silica capture method. Citric buffer, lithium chloride, and ames buffer methods did not work properly in extracting nucleic acids and they did not show any bands formations on agarose gel after running the RT-PCR products in the electrophoresis. The best option for extracting nucleic acids from plant pathogenic viroids may depend on several factors such as the nature of the sample, the downstream application, and the sensitivity of the detection method.

The TNA extraction method has a highly significant impact on the constitution of RT-PCR final productions. The results indicated that the silica capture method provided suitable for extracting nucleic acids from plant tissues. Sipahioglu et al. (2007) also obtained very good results with the silica capture method based on Foissac et al. (2000), who reported that nucleic acid extraction was realized at the highest rate and quality with the silica capture method. That result was also confirmed by our study and the best result was obtained with this method. As compared to the other three extraction methods, this method was simple and fast and had a high yield of purified nucleic acids. Positive results could not be taken with the other three extraction methods tried. In addition, it was seen that this method was fast and easy to perform when compared to the others.

Although positive results were obtained from the lithium chloride method in some studies (Cieslinska 2004, Hataya et al. 1999, Loreti et al. 1999, Ragozzino et al. 2003, Shamloul et al. 2002), we could not get positive results with this extraction method. This may be derived from its requiring special handling of the RNA pellet, particularly during the washing steps. In the same way, certain types of RNA, including viral RNA, may not be as effective using this method. Navarro et al. (2000) confirmed our results in their study. They reported that lithium chloride was less effective than other methods for isolating viroid RNA from infected plants (Navarro et al. 2000). On the contrary, Cieslinska (2004) reported that even diluted ratios of the lithium chloride method were more successful and efficient than the silica capture method in obtaining strawberry mottle virus RNA in strawberry tissues. This may depend on several factors, such as the nature of the sample, the downstream application, and the sensitivity of the detection method. However, the silica capture method as mentioned above was found to be more suitable for extracting nucleic acids from plant tissues in many plant virology studies (Rott and Jelkmann 2001, Sipahioglu et al. 2007, Zacharzewska et al. 2014)

The ames buffer method has the advantage of being the fastest method for the total nucleic acid extraction process compared to the other nucleic acid extraction methods (Laulhere and Rozier 1976, Podleckis et al. 1993). However, the purity of the RNA produced by the process is just as important as how long it took. However, in our study, we could not get good results with that extraction method.

It seems perfectly reasonable that the citric acid method we used in our study did not yield positive results. Because it was noted that the citric buffer method is a more practical way to isolate DNA from small amounts of fresh plant tissue (Doyle and Doyle 1987). Although it was reported to successfully isolate viroid RNA from infected plant tissues (Diener and Lawson 1984), it is particularly useful for extracting DNA from plant tissues. Therefore, it can be said that the citric buffer method may not be as effective at isolating RNA, and it may not be appropriate for all kinds of materials. Additionally, it was thought that these two methods deactivate the phenolic and polysaccharide compounds in the plant during nucleic acid extraction and reverse transcriptase. Furthermore, these compounds inhibit detection in RT-PCR tests. The nucleic acid used for the detection of viroids by PCR should be as pure as possible. Wilde et al. (1990) proved that reverse transcriptase with its over-sensibility has a great significance on the appearance of the inhibitive and regulative characteristics of the substances in the amplification reactions. This also demonstrates that the extraction methods used in the study played a significant role in removing the inhibitory substance. The usage of silica capture minimizes the deterioration of PCR products. RNA isolated and PCR products formed by the silica capture method can stay without spoiling for a couple of months and can be kept to work on it again. This shows the existence of a very useful method for long-term analysis of both plants and products and as well as for PCR products and RNA-isolated from them (Sipahioglu et al. 2006a).

This shows how the reverse transcription stage is important while working with viroids on RT-PCR and how the phenolic compounds existing in plant tissues can affect the result of extraction high in a contrary way.

The lithium chloride and the silica capture methods which contain mercaptoethanol can minimize the effect of polyphenols and polysaccharides in plant tissues thus both methods can work on various plant species in the extraction of nucleic acids very well (Cieslinska 2004, Sipahioglu et al. 2006b). After getting the results in that variation, it is obvious that how silica capture method is the correct method in use for nucleic acid extraction. Our study showed that the silica capture method is the most reliable and it can be used successfully in the detection of PLMVD and HSVd on stone fruits. The study also showed that using different methods for distinct viroids on various plant species can show dissimilar results like the success of the silica capture method and failure of the lithium chloride, citric acid, and ames buffer method. Obtaining results by using these methods can be very fruitful from the perspective of today's fruit-growing sector, in the production of rootstock, sapling, and grafting materials for obtaining disease-free and certified production materials.

In conclusion, the silica capture method appears to be the best option for isolating total nucleic acids of PLMVd and HSVd from stone viroids. However, when choosing an extraction method, it is crucial to keep in mind that the particular type of viroid and the characteristics of the sample being analyzed may have an impact on the choice of extraction method. Finally, this study revealed once again that the silica capture extraction method is the most suitable extraction method, which ensures that the nucleic acid of plant pathogenic viruses and viroids is obtained in the highest yield, purest, and without causing any loss or degradation.

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ÖZET

Sert çekirdekli meyve ağaçlarında ekonomik zarara neden olan peach latent mosaic viroid (PLMVd) ve hop stunt viroid (HSVd)'leri RT-PCR ile tanılamak için 4 farklı nükleik asit ekstraksiyon yöntemi kullanılmıştır. Yaprak örnekleri İzmir ilinde farklı meyve bahçelerinden viroid benzeri belirti gösteren ağaçlardan alınmıştır. Silica capture, lithium chloride, citric buffer ve ames buffer yöntemleri kullanılarak total nükleik asit ekstraksiyonu gerçekleştirilmiştir. Dört farklı ekstraksiyon yöntemi erik, kayısı ve şeftali ağaçlarından farklı dönemlerde toplanan örnekler kullanılarak birbiri ile karşılaştırılmıştır. Altmış dört farklı sert çekirdekli bitki örneği RT-PCR test yöntemi ile değerlendirilmiş ve silica capture ekstraksiyon yöntemi, kullanılan yöntemler arasında en güvenilir sonucu vermiştir. Diğer 3 ekstraksiyon yöntemi ile yapılan ekstraksiyonlarda istenilen sonuç alınamamıştır.

Anahtar kelimeler: TNA ekstraksiyonu, RT-PCR, viroid, sert çekirdekli meyve, nükleik asit

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