USE OF RT-PCR TECHNIQUES IN DETECTION OF VIRUSES OF PEPPER IN REPUBLIC OF MACEDONIA

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Abstract
Plant viruses can be a limiting factor and cause economically significant damage in the agricultural production. That is why, early and precise detection is very important in prevention of significant yield losses. In this study, early detection of plant viruses was performed on pepper, as one of the economically most important crops in Republic of Macedonia. Pepper plants were tested for *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV) and *Potato virus Y* (PVY), most common RNA viruses in pepper production. Virus antigen accumulation was measured with the use of DAS-ELISA method on 259 plant samples. CMV was detected as most prevalent virus on pepper (51% in 2012, 34% in 2013 and 61% in 2014). In 2012, AMV was detected as second, while in 2013 and 2014 was PVY. Detection was performed using Reverse Transcription – Polymerase Chain Reaction (RT-PCR). The RNA was extracted using TRIzol method from the collected leaf samples. RT-PCR was performed for each of the viruses separately due to the differences in the annealing temperatures of the used primers. In order to prevent RNA degradation of the plant viruses, each step of manipulation with the plant material was carefully optimized. The gained amplicons after RT-PCR, associated with different viruses were: for CMV the amplicon was 773 bp, for AMV was 669 bp, while for PVY 902 bp, respectively. The RT-PCR method exhibited high sensitivity, detecting the viruses in the early stages of infection, before observation of any visible symptoms on the pepper plants and measuring negative OD absorbance by DAS-ELISA.

Key words: molecular detection, CMV, AMV, PVY.

Introduction

Pepper (*Capsicum annuum* L.) is one of the most important cultivated crops in the Republic of Macedonia (Jankulovski, 1997) with production amongst the top ten countries in Europe (FAO, 2015). Plant viruses represent a major problem and a limiting factor in pepper production (Jovanchev et al., 1996; Rusevski and Bandzo, 1998). The most common pepper viruses are: *Cucumber mosaic virus* - CMV, *Alfalfa mosaic virus* - AMV, *Tobacco mosaic virus* - TMV, *Tomato spotted wilt virus* - TSWV, Potato virus *X* and *Y* - PVX and PVY **(**Jovanchev et al., 1996; Milošević, 2013). These pathogens can cause damage in pepper production up to 100% (Šutić, 1995; Jovanchev et al., 1996). Symptom manifestation of plant virus infection depends on various factors, amongst which are weather conditions, overall condition of the plant itself, susceptibility of the pepper variety, virus virulence (Šutić, 1995), as well as time of virus infection and presence of mixed infections caused by two or more viruses (Kim et al., 2010). Because of these factors, symptoms may vary from typical, to masked or atypical symptoms (Nair et al., 2009). Because of that, visual detection of virus infections has only a preliminary role. Although some viruses, such as AMV, cause specific symptoms, still virus determination based solely on visual observation is not sufficient. Symptoms may point to general virus infection, while for a final diagnosis and virus determination, laboratory tests should be performed. For fast detection of the inspected viruses, usually serological methods, which detect the virus on a protein level, such as Double Antibody Sandwich – Enzyme Linked Immunosorbent Assay (DAS-ELISA), are performed.

They are relatively fast, robust and simple with satisfactory level of sensibility (Alonso-Prados et al., 2003; Chatzivassiliou et al., 2004). Comparing the serological with the molecular methods, the latter are distinguished with a greater sensitivity (Raj et al., 2002; Niimi et al., 2003). Because of that, in order to gain more accurate results, molecular methods such as Polymerase Chain Reaction (PCR) and Reverse Transcription – Polymerase Chain Reaction (RT-PCR) for RNA viruses are performed (Nie and Singh, 2000; Lee and Ryu, 2009).
This study was succeeding previous findings of virus occurrence on pepper in R. Macedonia (Rusevski et al., 2009; 2010; 2011; 2013), promoting molecular methods as a means of detection of plant viruses in the agricultural production.

Material and methods

Sample collection

A total of 259 pepper plant samples (91 sample in 2012, 84 samples in 2013 and 84 samples in 2014) were collected after visual inspection at 13 different localities from eight pepper production regions in R. Macedonia: Skopje, Kumanovo, Sveti Nikole, Kochani, Strumica, Radovish, Prilep and Bitola. Samples were collected from symptomatic plants showing general virus symptoms, such as: bright yellow to white mosaic, chlorosis and deformation on leaves, plant tissue necrosis and stunted growth. In order to perform isolation and testing of the inspected viruses (AMV, PVY and CMV), pepper leaves were collected from the upper parts of the plant. The samples from the field were brought to the laboratory by placing them in liquid nitrogen, in order to prevent RNA degradation (de Wijs and Suda-Bachmann, 1979) and later on were stored at -80ºC until tested (Chen et al., 2011; Wang et al., 2012).

Serological analysis

The presence of the inspected viruses was determined on the collected leaf samples tested by DAS-ELISA, as described by Clark and Adams (1977) and modified and proposed by Bioreba AG (Wernli, 1999), using commercial polyclonal antisera. Plant tissue samples were homogenized in extraction buffer (1:10 w/v). The tested samples were considered to be positive if the average optical density (OD) value after one hour incubation at room temperature in the dark was at least twice higher than the average OD of the negative control. Measures were done on ELISA microplate reader MULTISCAN ASCENT at the wavelength of 405 nm.

Molecular tests

Total RNA was extracted from 30 selected samples, using TRIzol® Reagent (Ambion, Life Technologies) according to manufacturer's instructions (Xu and Nie, 2006; Chen et al., 2011). To prevent RNA degradation, homogenization of the plant material was performed in liquid nitrogen (Bertolini et al., 2003). RT was performed in a total volume of 20 µl reaction mixture consisting of 3µl of total RNA, 2µl 10xPCR Buffer Gold, 4µl $MgCl_2$ (25mM), 8µl dNTP's (2.5mM), 1µl (50pM/µl) of reverse (antisense) primer R, 1µl RNase Inhibitor and 1µl of MuLV Reverse Transcriptase (Applied Biosystems, USA). Detailed review of primers used, their sequence and amplicon sizes is given in Table 1. The RT was performed according to van Dongen et al. (1999).

Table 1. Primers used in RT-PCR for amplification of the capsid protein gene of CMV, AMV and PVY

Primer	Sequence (5' to 3')	Amplicon size (bp)	Reference
F2 CMV	ATGGACAAATCTGRATCWMCC	773	Deyong et al. (2005)
R2 CMV	CTGGATGGACAACCCGTTC		
F2 AMV	ATCATGAGTTCTTCACAAAAGAA	669	Xu and Nie (2006)
R2 AMV	TCAATGACGATCAAGATCGTC		
F2 PVY	CTAAGAAGCTTCACTGAAATG	902	Llave et al. (1999)
R2 PVY	ATATCGGATCCGGAGAGACAC		

The primer pair for CMV covered the capsid protein (CP) gene and part of the 3' NTR (3' non translated region). The set of primers for AMV included CP gene, while primer pair for PVY covered CP gene, part of the 3' end of the gene for NIb (Nuclear Inclusion protein b) and the beginning of the 3' NTR. The primers were chosen from conservative RNA regions of the inspected viruses. PCR was done in 25µl mixture volume, which contained 5µl cDNA, 2.5µl 10xPCR Buffer II, 2.5µl $MgCl_2$ (25mM), 2µl dNTP's (2.5mM), 0.5µl (100pM/µl) of forward (sense) primer F (Table 1), 0.5µl (100pM/µl) of reverse (antisense) primer R and 0.25µl of Taq DNA Polymerase (Sigma-Aldrich, USA). The PCR protocol for amplification of the CP gene for CMV was customized to cycling conditions specific for the CMV primer pair: initial melting at 95°C for 15 min, 35 cycles of 95°C for 45 s (melting), 59°C for 45 s (annealing) and 72°C for 90 s (extension) and final extension at 72°C for 10 min. The PCR for AMV was performed according to Xu and Nie (2006), and for PVY as described by Llave et al. (1999). RT-PCR was performed on thermocycler Techne, TC – 512 (Fisher Scientific, USA). Two types of negative controls were prepared, where cDNA was omitted and where cDNA from uninfected (ELISA-negative) leaf samples was used. The extracted RNA from pepper (its 18S rRNA used as internal control of RNA extraction quality) and the amplified products were analyzed on 1.5% agarose gel electrophoresis, in 1xTBE buffer and visualized under a UV transilluminator (Popovski et al., 2013).

Results and discussion

Virus occurrence determined by ELISA

After performing DAS-ELISA on the collected leaf samples, CMV was detected as most prevalent virus on pepper plants in R. Macedonia (51% in 2012, 34% in 2013 and 61% in 2014) (Table 2). In 2012, AMV was detected as second (15%), while in 2013 and 2014 it was PVY (7% and 8%, respectively). Plant viruses regularly occur on pepper plants throughout the vegetation in R. Macedonia (Jovanchev et al., 1996; Rusevski et al., 2011; 2013) and other countries (Choi et al., 2005; Kim et al., 2010; Milošević, 2013), causing economic losses and representing a major threat for pepper production. The frequency of CMV on pepper was observed by other authors (Choi et al., 2005; Ormeño et al., 2006; Kim et al., 2010).

Table 2. Incidence of *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV) and *Potato virus Y* (PVY) on pepper plants in R. Macedonia in the period of 2012-2014

Year	Number of tested samples	Detected viruses		
		CMV	AMV	PVY
2012	91	46 (51 %)	14 (15 %)	5 (5 %)
2013	84	28 (34 %)	2 (2 %)	6 (7 %)
2014	84	51 (61 %)	2 (2 %)	7 (8 %)

Molecular detection of AMV

After optimization of the RT-PCR protocol, because of the small virus occurrence of AMV during the tested period, a specific AMV isolate KUA7-2013 was chosen. The primer set AMV-F2/R2 was used for amplification of the entire region of the CP gene, generating amplicon of 669 bp (Figure 1). Due to change of conductivity, which can be a result of the amount of salts in the restriction buffer, or the salt constitution of the marker dyes, the size standard and the obtained fragment ran differently. Too much salt made the sample run slower, so fragment looked larger. The problem can be solved by staining the gel after electrophoresis, which we intend to do in the future, so next time this kind of discrepancies should be avoided. In the negative control, no amplification product was observed.

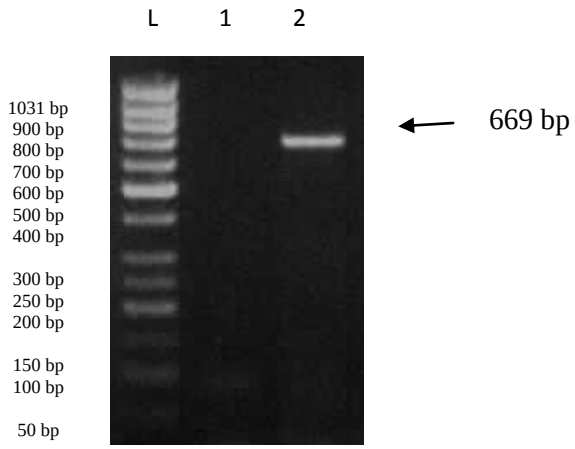

Figure 1. 1.5% agarose gel electrophoresis (AGE) analysis of the amplicon of the CP gene of *Alfalfa mosaic virus* (AMV) obtained by RT-PCR. L – GeneRuler™ 50bp DNA Ladder (Fermentas Life Sciences GmbH, Lithuania); lane 1 – negative control; lane 2 – isolate KUA7-2013.

The isolate KUA7-2013 was obtained from the area around Kumanovo in 2013. This particular isolate was chosen because of its unusual serological behavior. During the first test (performed after planting of the seedling material on field), a very high OD absorbance was measured (2.575), but during the second test (during flowering) a negative serological result was obtained (0.104). After performing a successful RNA isolation and RT-PCR, an amplicon of 669 bp was distinctly observed (Figure 1), showing that it is not a case of false positive serological result. This kind of fluctuations of the OD absorbance in AMV and some other plant viruses were observed also by other authors (Jaspars and Bos, 1980; Rusevski et al., 2013). With the help of RT-PCR, the problem was resolved, displaying the importance of the sensitivity of used methods.

Molecular detection of CMV

For the RT-PCR detection of CMV, ten leaf samples were chosen, having different concentrations of virus accumulation represented by OD values measured with DAS-ELISA and showing various symptom visibilities.

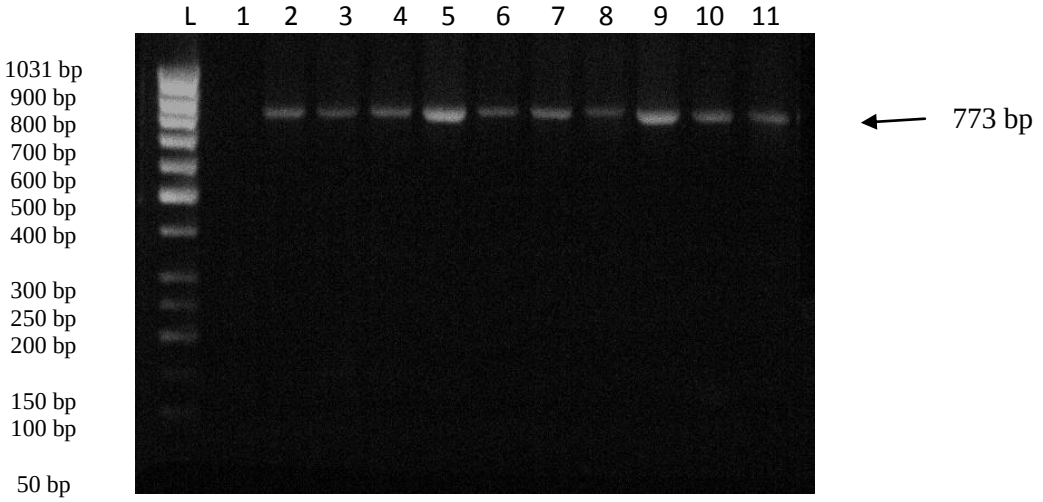

Figure 2. 1.5% agarose gel electrophoresis (AGE) analysis of the amplicon of the CP gene of *Cucumber mosaic virus* (CMV) obtained by RT-PCR. L – GeneRuler™ 50bp DNA Ladder (Fermentas Life Sciences GmbH, Lithuania); lane 1 – negative control; lane 2 – isolate SVNA3-2013; lane 3 – isolate SVNA6-2013; lane 4 – isolate SVNA7-2013; lane 5 – isolate SKA5-2013; lane 6 – isolate SKA3-2012; lane 7 – isolate KUA4-2014; lane 8 – isolate KUA7-2013; lane 9 – isolate STRA4-2013; lane 10 – isolate RAA6-2013 lane 11 – isolate SVNA3-2014.

The presence of CMV was positively identified in all of the tested samples, obtaining amplicons of the complete CP gene with the size of 773 bp. As it was in the case of the electrophoresis of AMV mentioned above, due to change of conductivity, the size standard and the obtained fragments ran differently. Too much salt made the samples run slower, so fragments looked larger. In the negative control, no amplification product was observed (Figure 2). RT-PCR was widely used for CMV detection (Vučurović et al., 2010; Fidan and Güllü, 2014) and amplicons were detected even in plant samples where measured OD absorbance (virus concentration) was very low (Yu et al., 2005; Chen et al., 2011).

Molecular detection of PVY

The presence of PVY was positively identified by the use of RT-PCR, obtaining amplicons of the complete CP gene with the expected size of 902 bp. In the negative control, no amplification product was observed (Figure 3).

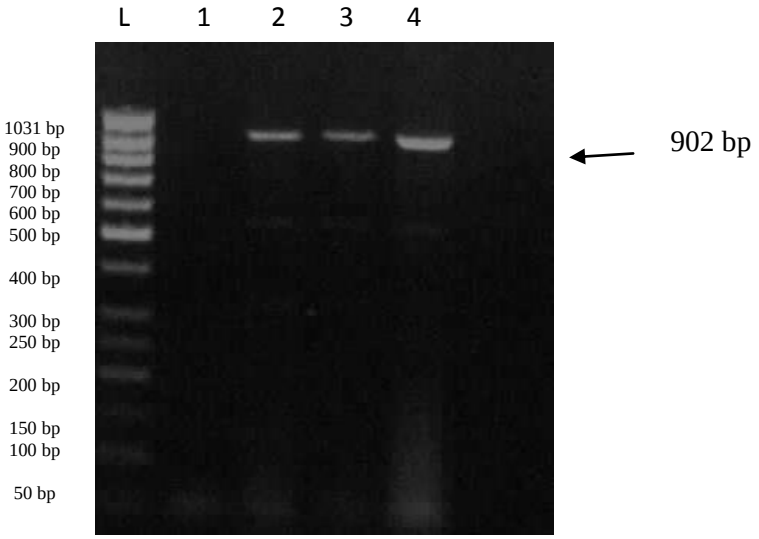

Figure 3. 1.5% agarose gel electrophoresis (AGE) analysis of the amplicon of the CP gene of *Potato virus Y* (PVY) obtained by RT-PCR. L – GeneRuler^{TM} 50bp DNA Ladder (Fermentas Life Sciences GmbH, Lithuania); lane 1 – negative control; lane 2 – isolate KUA6-2013; lane 3 – isolate KUA7-2013; lane 4 – isolate KOCB1-2013.

The PVY-isolates detected by RT-PCR were chosen as representatives of different types of infection. The isolate KUA6-2013 was part of a single infection, the isolate KUA7-2013 was part of a triple mixed infection (AMV+CMV+PVY) and the isolate KOCB1-2013 was part of a double mixed infection including CMV. After performing RT-PCR, bands of all amplicons with size of 902bp were clearly visible (Figure 3), regardless the type of infection. This proves again the sensitivity and the selectiveness of the method (Nie and Singh, 2000; Wang et al., 2012).

Conclusions

With the RT-PCR analysis performed during this trial on isolates of the inspected pepper plant viruses (AMV, CMV and PVY), the sensitivity and exclusivity of the method was confirmed. The type of infection (single or mixed), or the concentration of the virus accumulation in the plants represented by different OD values have not affected the accuracy of RT-PCR. Because of the obvious benefits from the use of the molecular methods, we hope that RT-PCR will take its rightful place in plant virus detection and controlling of the virus occurrence in the agricultural production in R. Macedonia.

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