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Detection of viruses in seeds of some vegetables by reverse transcriptase polymerase chain reaction (RT-PCR)

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The present study was carried out to detect the presence of Alfalfa mosaic alfamovirus (AMV), cucumber mosaic cucumovirus (CMV), lettuce mosaic potyvirus (LMV), cucumber green mottle mosaic virus (CGMMV), tomato bushy stunt tombusvirus (TBSV), tobacco mosaic tobamovirus (TMV), tomato black ring nepovirus (TBRV) and tomato mosaic tobamovirus (ToMV) in seeds of pepper, tomato, cucumber and lettuce which are essential for human nutrition and seed production in Turkey. 50 seed samples for each vegetable obtained from various foundations and farmers were tested by reverse transcriptase polymerase chain reaction (RT-PCR) and double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was conducted to compare the results. Besides, two total nucleic acid (TNA) extraction methods and a commercial RNA purification kit was compared to get high quality RNA for optimized RT-PCR. Purified total RNA extracts were used as template for cDNA synthesis, PCR products were analyzed by gel electrophoresis, and visualized by ethidium bromide staining. As a result of all, the optimum TNA extraction method was silica capture which is practical and easy to use for getting viral agents from tested vegetable seeds. RT-PCR was found rapid and sensitive in detecting viruses in seeds of vegetables when compared to DAS-ELISA. This study shows that RT-PCR can be successfully applied in certification programs and quarantine tests of vegetable seeds.

Key words: Seed, reverse transcriptase polymerase chain reaction (RT-PCR), total nucleic acid (TNA) extraction, optimization.

INTRODUCTION

Seed is one of the most important inputs in crop production since crop statues substantially associate with the seed material for sowing. Based on the fact that being starting and crucial material of production, it is a key factor in contributing to increase in yield. Although other cultivation activities such as tillage, irrigation, fertilization and plant protection were carried out precisely, getting sufficient production depends on quality of used seeds (Şehirali, 1989). Seed health and vigor play an important role for growing healthy and quality crops. Seed-borne pathogens may cause serious damage in quality and death of plants, resulting in loss of crops and foods.

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Abbreviations: AMV, Alfalfa mosaic alfamovirus; **CMV,** cucumber mosaic cucumovirus; **LMV,** lettuce mosaic potyvirus; **CGMMV,** cucumber green mottle mosaic virus; **TBSV,** tomato bushy stunt tombusvirus; **TMV,** tobacco mosaic tobamovirus; **TBRV,** tomato black ring nepovirus; **ToMV,** tomato mosaic tobamovirus; **DAS-ELISA,** double-antibody sandwich enzyme linked immunosorbent assay; **RT-PCR,** reverse transcriptase polymerase chain reaction; **TNA,** total nucleic acid.

The world's seed trade has developed quickly due to increased cooperation between countries and improved transportation facilities (ISF, 2011). Due to this, many problems have been revealed, such as the spreading of plant diseases throughout the world. Diseased seeds are responsible for spreading and transporting disease agents all around the world (Neergaard, 1988; Mink, 1993; Crowley, 1957).

Viruses are one of the most important disease agents in vegetable seeds and cause serious problems such as reduction in yield and germination, changes in shape and color of seeds (Hemmati and McLean, 1977; Mandhare and Gawade, 2010; Inouye, 1962). Seed-borne viruses also have indirect effects, such as the cost of chemicals applied to virus vectors and virus-free production materials except direct effects mentioned above (Hull, 2009). Besides, the lack of chemical management of viral agents, in addition to producer's insufficient level of knowledge regarding the viruses, makes it difficult to prevent economic losses (Erkan, 1998). In addition, the emergence and severity of virus diseases can vary according to virus, host, vector and environmental conditions rectangle relationships (Allam et al., 1980; Tosic et al., 1980).

Approximately, 18 to 20% of known plant viruses are seed transmittable (Johansen et al., 1994). Viral agents are important for source of diseases at the beginning of production even at low rates of seed transmission (Nienhaus, 1976). In addition, seed-borne viruses can exploit the other transmission methods and it causes disease to spread quickly. Seed transmission is used as a method between different vegetation periods, despite the low percentage of seed transmission (Erkan, 1998). Studies conducted in the years past have demonstrated that virus infected seeds can be found easily in every part of the world, even if they are certified seeds (Albrechtsen, 2006; Paylan, 2011).

Considering all these issues, the importance of seed health has been understood; thanks to previous studies that have been conducted (Agarwal and Sinclair, 1997; Maude, 1996). For these diseases caused by viral agent, using healthy and virus-free production material is the most important measure. The determination of viral agents on seeds forms the basis of protection against these diseases. In order to identify viruses on seeds, commonly used methods are the serological test and dyeing (Franken et al., 1990; von Wechmar et al., 1984; Ojuederie et al., 2009). Enzyme linked immunosorbent assay (ELISA) which is, because of its many advantages, the most commonly used serological method (Porstmann and Kiessig, 1992). However, the accuracy and reliability of molecular methods is a fact beyond dispute.

In this study, our aim was to determine the presence and prevalence of alfalfa mosaic alfamovirus (AMV), cucumber mosaic cucumovirus (CMV), lettuce mosaic potyvirus (LMV), cucumber green mottle mosaic virus (CGMMV), tomato bushy stunt tombusvirus (TBSV), tobacco mosaic tobamovirus (TMV), tomato black ring nepovirus (TBRV) and tomato mosaic tobamovirus (ToMV) in some vegetable seeds by reverse transcriptase polymerase chain reaction (RT-PCR). In addition, the nucleic acid extraction method and common identifying methods were compared and RT-PCR optimization studies were carried out. Optimization studies for both nucleic acid extraction and RT-PCR and updated data that shows occurrence of viruses in essential vegetables for Turkey made this publication different from others.

MATERIALS AND METHODS

Seed material

Pepper (*Capsicum annuum* L.), tomato (*Solanum lycopersicum* L.), melon (*Cucumis melo* L.), lettuce (*Lactuca sativa* L.) seeds obtained from various foundations and farmers. 50 seed samples for each vegetable were prepared according to ISTA rules (Anonymous, 2004).

Double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) method

The DAS-ELISA method was applied using monoclonal antibodies as Clark and Adams (1977) and Erkan et al. (1995) have determined. Commercial antibodies (IgG) obtained from firms (Agdia, USA; Bioreba, Switzerland; Loewe, Germany) were diluted in coating buffer as per dilution rates and 200 µl was added to each well. Then the plates were incubated for 4 h at 37°C and washed 3 times with PBS-Tween. Seeds were macerated in the extraction buffer and crushed by pestle. 200 µl extracted seed samples was added to each well and incubated for overnight at +4°C. The washing stage with PBS-Tween was applied again and 200 µl diluted conjugated IgG was added to each well and plates were incubated for 4 h at 37°C. After this period, plates were washed again and 200 µl was added to each well from 1 mg/ml paranitrophenyl phosphate, including a substrate buffer, before being incubated at room temperature. Evaluation was made by monitoring the yellow color formation using the naked eye and read spectrophotometrically on the ELISA-reader at 405 nm.

Reverse transcriptase polymerase chain reaction (RT-PCR) method

Economically important vegetable seeds (pepper, tomato, cucumber and lettuce) in our country were used as a material and tested by RT-PCR (Table 1). The total nucleic acid (TNA) extraction step was conducted using Foissac et al. (2001)'s "silica capture" method, "citric buffer method (Wetzel et al., 1992) and "GeneJET Plant RNA Purification Mini Kit" protocol (Fermentas, USA).

Silica-capture method

The silica-capture isolation was carried out according to Foissac et al. (2001). For this, 1 g seed sample from each sample was measured and placed in plastic bags. Then, they were homogenized with 2 ml grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc, 2.5% w/v PVP-40, 25 mM EDTA, 1.0 KOAc, 1% 2-mercaptoethanol). 500 µl extract was transferred to

Table 1. Vegetable seeds tested by RT-PCR method and viral agents tested in these seeds.

Parameter	Pepper	Tomato	Cucumber	Lettuce
AMV	+			
CGMMV			+	
CMV	+		+	
LMV				+
TMV	+	+		
TBRV		+		+
TBSV		+		
ToMV	+	+		
Sample number	50	50	50	50

new tubes involving 100 ml of 10% sodium lauryl sarcosyl solution and they were mixed with each other. After, they were incubated at 70°C for 10 min and in ice for 5 min, centrifuged at 14000 rpm for 10 min. Three hundred (300) ml of the supernatant was transferred to a new eppendorfs containing 150 ml of ethanol, 100 ml of resuspended silica and 300 ml of 6 M sodium iodide. After that, the mixture was incubated at room temperature on shaker, tubes were centrifuged at 6000 rpm for 1 min, and obtained pellets were washed twice with washing buffer. Besides, 150 µl nuclease free sterile water was added to tubes and they were centrifuged at 14000 rpm for 3 min. The supernatant was transferred to new tubes and kept at –20°C until used.

Citric buffer method

One gram seed samples were homogenized with 1 ml citric buffer (containing 50 mM sodium citrate, 20 mM DIECA, 2% PVP) and some quartz sand. The 50 µl supernatant obtained from centrifugation at 8000 rpm for three min was collected in a new tube and 450 µl of citric buffer was added to in question tube. They were kept at -20°C (Wetzel et al., 1992; Sipahioğlu et al., 2007).

Gene JET plant RNA purification mini kit protocol

1 g seed samples were homogenized with 500 µl of plant RNA lysis solution added 10 µl 2 M dithiothreitol (DTT). After, they were centrifuged at 56°C for 3 min following incubation at 14000 rpm for 5 min; supernatant was transferred to new tubes involving 250 µl of 96% ethanol and mixed. They were centrifuged at 11000 rpm for 1 min, flow-through solution discarded and the membrane of purification tube was washed with 700 µl washing buffer 1 and two times 500 µl washing buffer 2, respectively. At the end of these steps, 50 µl nuclease free sterile water was added to tubes and total nucleic acid was collected in tubes with centrifugation at 11000 rpm for 1 min. They were kept under -20°C until used (Fermentas, USA). Samples were extracted as mentioned above and cDNA synthesis was carried out using the First Strand cDNA Synthesis Kit (Fermentas, USA). The RT-PCR specific primers of each virus and the specific conditions are presented in the Table 2. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

RESULTS

DAS-ELISA

The results of the ELISA studies carried out in order to determine the presence of AMV, CMV, TMV and ToMV revealed the presence of CMV, TMV and ToMV infections on pepper seeds. However, AMV has not been isolated from any samples. The tomato seed samples were tested for TMV, TBRV, TBSV and ToMV infections. The test results reveal the presence of TMV and ToMV infections. In addition, CMV infections were detected on cucumber seeds and LMV on lettuce seeds.

Total RNA extraction results

TNA extraction results were compared and evaluated according to TNA gel pictures. Firstly, we observed TNA images in silica capture method and Gene JET plant RNA purification mini kit protocol with assessments on TNA gel pictures. However, no TNA image was observed in citric buffer method. The quality of TNA extracted with silica capture was significantly higher than commercial kit. In conclusion, commercial kit gave moderate results and citric buffer method failed in terms of qualified total RNA isolation (Figure 1).

RT-PCR

RT-PCR results showed TMV and ToMV infections exist on tomato seeds and these results show similarity with ELISA. There is no TBRV and TBSV infection in tomato seeds according to results obtained from both ELISA and RT-PCR. The results of the RT-PCR tests conducted on AMV, CMV, TMV and ToMV on pepper seeds revealed the presence of CMV (Figure 2A), TMV (Figure 2B) and ToMV (Figure 2C) infection. The expected fragment size was observed to be 600, 422 and 508 bp, respectively. The RT-PCR test for CGMMV and CMV infections on cucumber seeds and TBRV, LMV on lettuce seeds proved the presence of CGMMV, CMV infections on cucumber and TBRV, LMV on lettuce seeds.

DISCUSSION

In this study, the presence of viral infections was

Table 2. Description of primers and polymerase chain reaction (PCR) conditions.

Virus	Primer	Cycling condition	Amplified fragment
AMV	5'-GT GGT GGG AAA GCT GGT AAA -3' 5'-CAC CCA GTG GAG GTC AGC ATT -3' (Martínez-Priego et al., 2004)	1X (94°C 2 min) 35X (94°C/ 30 s, 54°C/30 s, 72°C/30 s) 1X (72°C 10 min)	700 bp
CMV	5'-CGCCCT GCAGTGGTCTCCTTTTGGAG -3' 5'-ACT CCA ACT GGC TCG TAT GG -3' (Nakazono-Nagaoka et al., 2005)	1X (92°C 2 min) 30X (94°C/60 s, 58°C/20 s, 75°C/60s) 1X (75°C 5 min)	600 bp
LMV	5'- AAG GCA GTA AAA CTG ATG -3' 5'- TTT ATA CTA CAG TCT TTA -3' (Zerbini et al., 1995)	1X (94°C 1 min) 35X (94°C/60 s, 55°C/120 s, 72°C/120 s) 1X (72°C 10 min)	800 bp
CGMMV	5'- GTT TCG CCT CAA AAT TCC -3' 5'- TCT AAA TAT GAC AAG TCG C -3' (Moreno et al., 2004)	1X (95°C 5 min) 35X (94°C/30 s, 55°C/30 s, 72°C/ 90 s) 1X (72°C 10 min)	359 bp
TBSV	5'- GCA AAC TGT GCA GAT GAC TGT G -3' 5'- CAC AGT CAT CTG CAC AGT TTG C -3' (Boonrod et al., 2004)	1X (95°C 5 min) 35X (94°C/60 s, 60°C/60 s, 72°C/120s) 1X (72°C 7 min)	620 bp
TMV	5'-GACCTGACAAAAATGGAGAAGATCT3' 5'- GAA AGC GGA CAGAAA CCC GCT G -3' (Jacobi et al., 1998)	1X (95°C 5 min) 40X (94°C/30 s, 50 °C/60 s, 72°C/60 s) 1X (72°C 7 min)	422 bp
ToMV	5'- CTC CAT CGT TCACAC TCG TTA CT 3' 5'- GATCTGTCAAAG TCT GAGAAA CTT C-3' (Jacobi et al., 1998)	1X (94°C 2 min) 35X (94°C/30 s, 62°C/45 s, 72°C/60 s) 1X (72°C 5 min)	508 bp
TBRV	5'- ATGGGAGAAGTGCTGG-3' 5'- AATCTTTTTGTGTCCAAC-3' (Le Gall, 1995)	1X (95°C 5min) 35X (94°C/60 s, 54°C/60 s, 72°C/120 s) 1X (72°C 7 min)	330 bp

determined in some vegetable seeds which are economically important in our country. The methods (DAS-ELISA and RT-PCR) used in this study, revealed similar results. In addition, the results of this study reveal that RT-PCR is more sensitive than ELISA as stated by Usta et al. (2005), Berniak et al. (2009) and Dang et al. (2009) as mentioned before. For instance, in a study about determination of viruses in tomato seeds by DAS-ELISA, RT-PCR and dsRNA analyses, ToMV was not detected by ELISA. On the contrary, this agent was determined by RT-PCR (Yilmaz et al., 2003). Again in another research, success of RT-PCR in detection of seed-borne viruses had been found 100%, while success of ELİSA showed 96% ratio (Paylan, 2011). Similarly in this research, some samples were found to be infected using the RT-PCR test, in contrast to the ELISA results. Differences of these methods as sensitivity were especially observed in cucumber and lettuce seeds. Based on the fact that CGMV on cucumber seeds and TBRV on lettuce seeds were determined by RT-PCR, they were not able to be detected by DAS-ELISA. This can be explained by RT-PCR's low concentrations delectability feature. Besides, RT-PCR method also is more convenient than other molecular methods in terms of routine tests due to the fact that it can test large numbers of samples simultaneously (Lee et al., 2011). In addition to this, quality of total nucleic acid and extraction methods is so essential for RT-PCR applications. In this study, success of silica-capture method was also exhibited on determination of seed-borne viruses in vegetable seed.

With this study, the seed-borne viruses of the

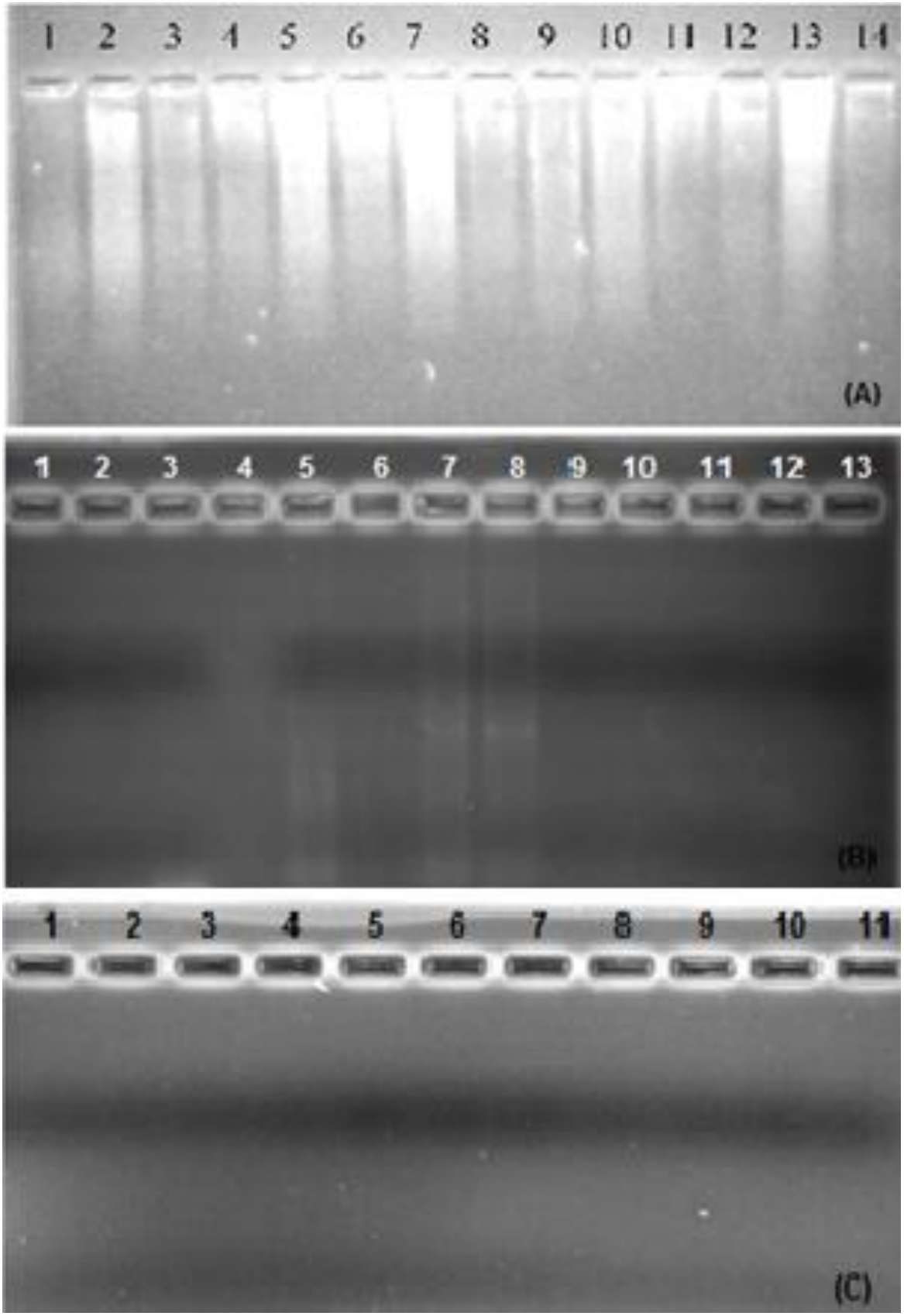

Figure 1. Agarose gel electrophoresis of isolated TNA samples with three different extraction methods. (A) Silica-capture method. (B) Gene JET plant RNA purification mini kit protocol. (C) Citric buffer method.

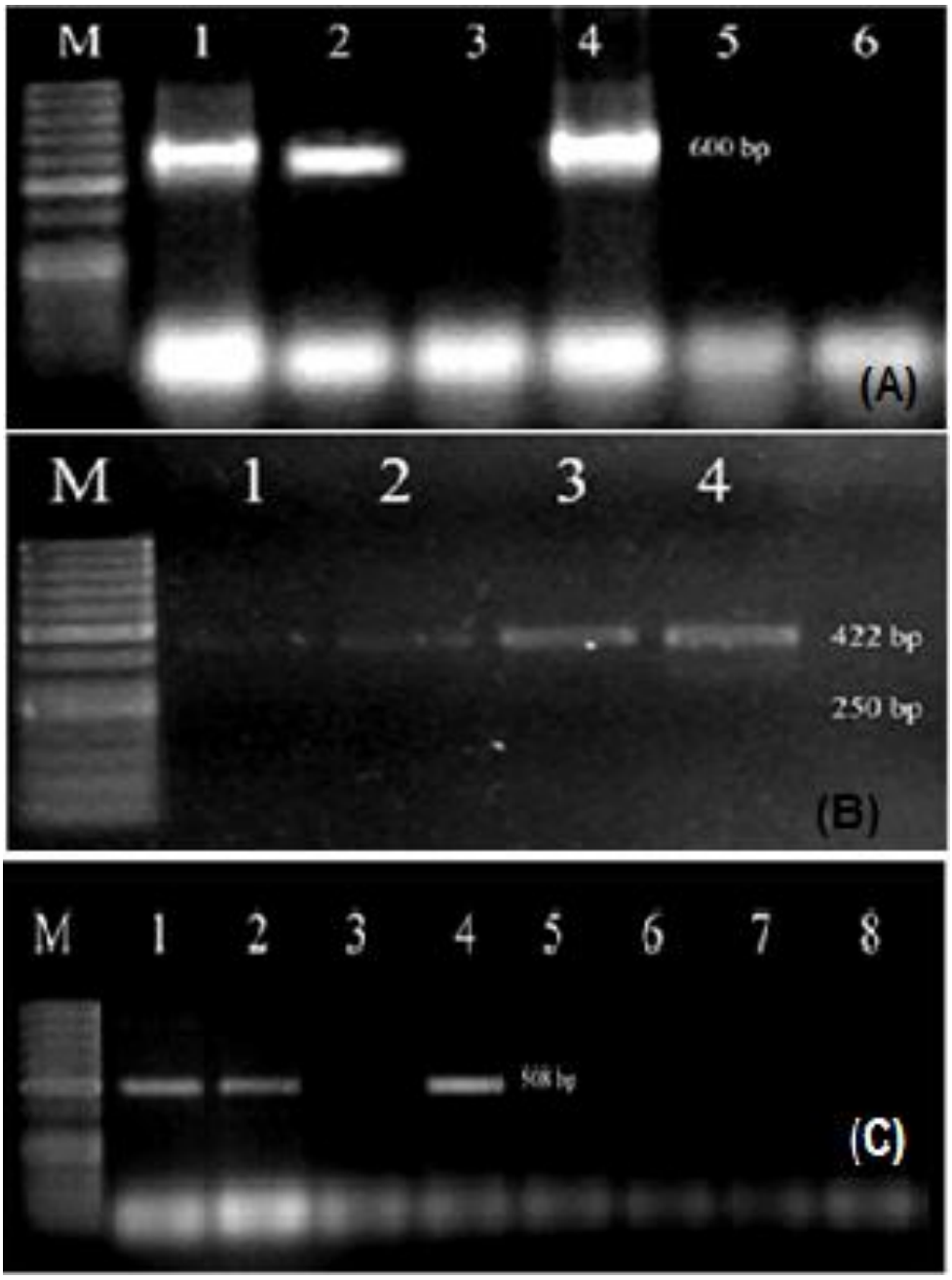

Figure 2. Agarose gel electrophoresis of RT-PCR products obtained from the detection of some viruses. A. RT-PCR results of CMV: Lane 1, Positive CMV control; Lanes 2 and 4, virus positives; Lanes 5 and 6, healthy plant; M, molecular size markers. B. RT-PCR results of TMV: Lane 1, Positive TMV control; Lane 2, 3 and 4, virus positives; M, molecular size markers. C. RT-PCR results of ToMV: Lane 1, 2 and 4 are virus positives; M, molecular size markers; Lanes 3, 5, 6, 7 and 8, healthy plant.

aforementioned vegetable seeds were determined and preliminary knowledge was obtained for further studies; in addition to RT-PCR optimization studies are being carried out. Healthy seed usage is the most important method for protecting the production of quality vegetables in our country. A rapid and sensitive method is needed in order to determine and prevent seed-borne agents spreading quickly, especially taking the developing seed trade into account. It is required to control production materials in terms of seed-borne disease agents in seed certification, and quarantine programmes should be established for bringing production materials to country

(Neergaard, 1986; Morrison, 1999). The results of this study show that the RT-PCR test should play an important role in these programmes. ELISA is a more preferable method for the detection of plant viruses than PCR because of its advantages of not requiring special training, in addition to economic reasons. However, RT-PCR should be generalized, especially in seed certification programmes, due to its superior features such as being fast, routine, and reliable in being able to detect viruses even in low concentrations (Jacobi et al., 1998).

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