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A Preliminary Study on Molecular Differentiation of Apple and Hazelnut Isolates of *Apple Mosaic Virus* (ApMV)

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ABSTRACT

In order to investigate molecular variability between hazelnut and apple isolates of *Apple mosaic virus* (ApMV), the coat protein genes of both isolates were amplified using a procedure involving reverse transcription-polymerase chain reaction (RT-PCR). PCR products (about 820 bp) were obtained by using total RNAs extracted from bean (*Phaseolus vulgaris* L.) plants infected with ApMV-apple and hazelnut isolates and covered the entire coding sequence of the ApMV-coat protein (CP). RT-PCR products belonging to both isolates were further analyzed in restriction fragment length polymorphism (RFLP) by enzymatic digestion. Analysis of restriction fragments using ten different enzymes revealed no polymorphism within apple and hazelnut isolates of ApMV. These results suggested that apple and hazelnut isolates of ApMV might have nucleotide homology in the CP gene region. However, further studies are necessary for a detailed analysis of the CP gene of ApMV isolates from different hosts and regions.

Key words: ApMV, RT-PCR, RFLP, coat protein, apple, hazelnut

INTRODUCTION

The family *Bromoviridae* constitutes five families, *Ilarvirus*, *Alfamovirus*, *Cucumovirus*, *Bromovirus* and *Oleavirus*. The genus *Ilarvirus* is divided into subgroups based on their serological characteristics. *Apple mosaic virus* (ApMV) is in subgroup III of the genus *Ilarvirus*, which also includes *Prunus necrotic ringspot virus* (PNRSV) and *Rose mosaic virus* (Rybicki, 1995). Natural hosts of ApMV include apple, rose, hazelnut, horse chestnut, raspberry, birch and hops (Rybicki, 1995), stone fruits (Caglayan and Gazel, 1998; Saade et al., 2000), raspberry (Baumann et al., 1982) and strawberry (Tzenatakis and Martin, 2005). ApMV contains three genomic (RNA 1, RNA 2 and RNA 3) and one subgenomic RNA (RNA 4). Sequence analysis of ApMV RNA 1 and RNA 2 (Shiel and Berger, 2000), ApMV RNA 3 (Shiel et al., 1995) and RNA 4 (Alrefai et al., 1994; Sanchez-Navarro and Pallas, 1994) were completed. ApMV RNA 1 is 3476 nucleotides in length and encodes a single large open reading frame (ORF), whereas RNA 2 is 2979 nucleotides in length and also encodes a single ORF (Shiel and Berger, 2000). ApMV RNA 3 is 2056 nucleotides in length and has two open reading frames (ORFs). The first ORF encodes the putative movement protein, but

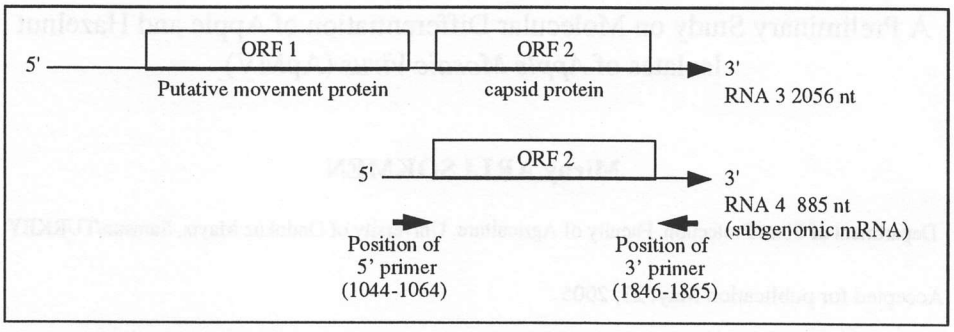

Figure 1. Genome organisation of ApMV RNA 3 (illustrated by using the sequence data from Alrefai et al., 1994 and Shiel et al., 1995). The position of capsid protein gene between nucleotides 1126 and 1794 in RNA 4.

the other translates coat protein (CP) by producing subgenomic mRNA (RNA 4) (Alrefai et al., 1994). ApMV RNA 4 contains an open reading frame (ORF) which encodes the CP of 25 056 daltons (Alrefai et al., 1994) (Figure 1). Coat protein of ilarviruses plays a significant role in initiation of infection (Sanchez-Navarro and Pallas, 1997) in addition to its property on viral RNA protection. Phylogenetic analysis of CP gene sequences of 17 ilarvirus isolates obtained from hop plants in Australia indicated that several ApMV isolate clusters distinct from PNRSV isolates (Crowle et al., 2003). The nucleotide sequence of the ApMV coat protein gene showed no similarity to those of viruses, such as *Alfalfa mosaic virus*, *Tobacco streak virus*, *Brome mosaic virus* or *Cucumber mosaic virus* in Bromoviridae family (Alrefai et al., 1994). On the other hand, the sequences of several ApMV CP genes published so far showed that the sequences of some isolates are highly variable (Alrefai et al., 1994; Guo et al., 1995; Shiel et al., 1995). The most recent study revealed that there were 6-15 additional nucleotides after nucleotide position at 141 in the CP gene when eight European isolates of ApMV from almond, apple, hop, prune and pear were compared with the sequences of the CP region of American isolates.

The CP gene of RNA viruses is usually variable and frequently used in phylogenic analysis (Dolja and Carrington, 1992; Petrzik and Lenz, 2002). Therefore, this study is planned to investigate genetic variability of hazelnut and apple isolates of ApMV on the basis of restriction analysis of CP gene derived by RT-PCR.

MATERIALS and METHODS

ApMV Isolates

Hazelnut isolate of ApMV was obtained in the previous study from Samsun Province (Arli Sokmen et al., 2004). Apple isolate of ApMV from Tokat province was kindly supplied by Dr. N. D. Kutluk Yilmaz (Department of Plant Protection, Faculty of Agriculture, University of Ondokuz Mayis, Samsun).

Propagation of ApMV Isolates

One apple and two hazelnut isolates of ApMV were propagated and maintained in *Phaseolus vulgaris* L. PI 212110 (the collection of Western Regional Introduction Station, Washington, the USA). ApMV-infected apple and hazelnut samples were homogenized in 0.02 M phosphate buffer (pH: 8.0) including 2% 2- mercaptoethanol and inoculated to *P. vulgaris* at 2-4 leaf stage (Fulton, 1972). After rinsing with tap water, plants were maintained in a growth cabinet at 20-22°C.

Extraction of Viral RNA

Total RNA was extracted from systemically infected leaves of *P. vulgaris* as described by Verwoerd et al. (1989). One to three leaf discs were collected and ground to a fine powder after cooling in liquid nitrogen. Heated (80°C) extraction buffer (0.1 M LiCl, 100 mM Tris-HCl pH: 8.0, 10 mM EDTA, 1% SDS: Phenol, [1:1]) was added to the ground powder and vortexed for 30 s. Supernatant was removed after chloroform: isoamylalcohol (24:1) treatment, and RNA was precipitated with 4 M LiCl by overnight incubation at 4°C and then high speed centrifugation at 13 000 g. The pellet was dissolved in RNase-free water and precipitated once more with two volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH: 5.2). Concentration of RNA was determined by considering that 1 A_{260} unit= 40 µg/ml for single-strand RNA.

Amplification of ApMV-CP Gene

One-step RT-PCR method was applied according to Manufacturer (Qiagen, Valencia, USA)'s instructions. Reverse transcription and PCR were carried out sequentially in the same tube by using the mixture of reverse transcriptase and *Taq* DNA polymerase enzymes. The reaction components were 400 µM of each dNTP and 0.6 µM of each primer in a final concentration, total RNA (2 µg/reaction), RNase inhibitor (10 Unite/reaction), Qiagen OneStep RT-PCR buffer and RNase-free water to complete the reaction volume of 50 µl. The downstream primer was complementary to the ends of the DNA fragment to be amplified (positions of 1846-1865 in ApMV RNA 3: 5'-ATG CTT TAG TTT CCT CTC GG-3'). Upstream primer had a sequence of 5'- GGC CAT TAG CGA CGA TTA GTC-3' (positions 1044-1064 in ApMV RNA 3) (Petrzik and Lenz, 2002) (Figure 1). RT-PCR conditions were one cycle of 30 min at 50°C (activation of reverse transcripatase), one cycle of 15 min at 95°C (denaturation of cDNA) followed by 35 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 68°C, and final incubation of 10 min at 68°C. Amplified fragments which contained the complete CP gene between nucleotide positions 1126 and 1794 were subjected to electrophoresis in a 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide.

Restriction Digestion of PCR Products

Restriction enzymes used for digestion of PCR products were *Hinf* I, *Mbo* I, *Hae* III, *Bam H* I from New England Biolabs (USA), *Hind* II, *Hind* III, *Eco RI*, *Msp* I, *Pst* I,

Xho I from Sigma (USA). Ten µl of PCR products from each isolate was directly cleaved with restriction enzymes (10 Unite/reaction) in a total reaction volume of 20 µl and incubated at 37°C for 2 hours with each enzymes The digestion products were resolved in 1% agarose gel or 4% QA-Agarose Multipurpose (Q-BIOgene, Irvine CA, USA), and the band patterns obtained were visualized and photographed on UV transilluminator.

RESULTS and DISCUSSION

Total RNA was extracted from leaves of the healthy and ApMV-infected plants usually 2-3 weeks after inoculation. PCR reactions were performed in order to amplify sequences between nucleotides 1126 and 1794 of the capsid protein gene of ApMV. PCR products of ApMV hazelnut and apple isolates had the size of predicted for the amplification product of about 820 base pair (bp) (Figure 2).

To obtain preliminary evidence for genetic polymorphism between ApMV isolates from hazelnut and apple, PCR products of each isolate were individually cleaved with restriction enzymes of *Hinf I*, *Mbo* I, *Hae* III, *Bam H* I, *Hind* II, *Hind* III, *Eco* RI, *Msp* I, *Pst* I, *Xho* I. Digested products (20 µl) were resolved in 1% agorose gel and visualized on UV transilluminator. Restriction endonuclease of Hae I digested the products from apple and hazelnut isolates and gave two bands (about 580 bp and 240 bp) (Figure 3). *Eco* RI and *Hinf* I were the other enzymes that cleaved the CP gene region of ApMV apple and hazelnut isolates and gave the bands of about 90 and 680 bp, and 90 and 420 bp, respectively. These enzyme digestions also included very small

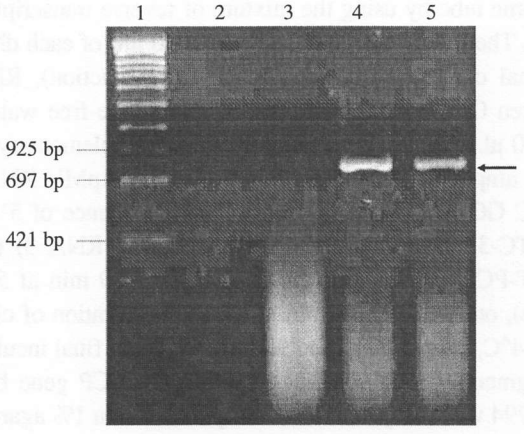

Figure 2. Amplification of the capsid protein gene of ApMV isolates maintained in *Phaseolus vulgaris* (PI 212110). Line 1: Marker (Lambda-pUC mix marker, MBI Fermantas); line 2: negative control (no total RNA included); line 3: total RNA extracted from healthy *P. vulgaris* plants; line 4: total RNA extracted from infected *P. vulgaris* with ApMV-apple isolate; line 5: total RNA extracted from infected *P. vulgaris* with ApMV-hazelnut isolate. Arrow indicates PCR product of about 820 bp.

bands of which molecular sizes could not be estimated. *Hind* III, *Xho* I, *Pst* I did not digest the RT-PCR products (Figure 3). The restriction enzymes of *Mbo* I, *Hind* II, *Msp* I and *BamH* I also did not digest the RT-PCR products of both isolates (data not shown). Analysis of restriction bands on the other gel containing 4% QA-Agarose Multipurpose (Q-BIOgene) for resolving the fragments smaller than 1 kb did not give satisfactory results (data not shown). Restriction analysis of PCR products showed no differences between ApMV isolates from hazelnut and apple according to ten different enzymes. The size and pattern of bands were identical for both isolates (Figure 3).

PCR-restriction fragment length polymorphisim (RFLP) is frequently used to reveal polymorphism within the same virus genus (Letschert et al., 2002) or within the same virus species (Accotto et al., 2000). To increase knowledge on biological diversity of PNRSV isolates, twenty five isolates with distinct symptom expression and originating from six different *Prunus* hosts were analyzed by RT-PCR-RFLP method and reported that. most of isolates were discriminated and clustered into three different groups (Aparicio et al., 1999). Twenty PCR-positive samples from different *Prunus* species growing in Turkey were subjected to RFLP analysis using eight different restriction enzymes, and the research revealed that most of the PNRSV isolates were members of group PV96, whereas a single isolate was a member of group PV32; none of the isolates belonged to group PE5. In this study, there was no relation between geographic distribution and grouping of PNRSV isolates (Ulubas and Ertunc, 2004). There is no information on serotypes and pathotypes of ApMV so far. However, the studies revealed that the aminoacid sequence of CP of the German ApMV isolate from

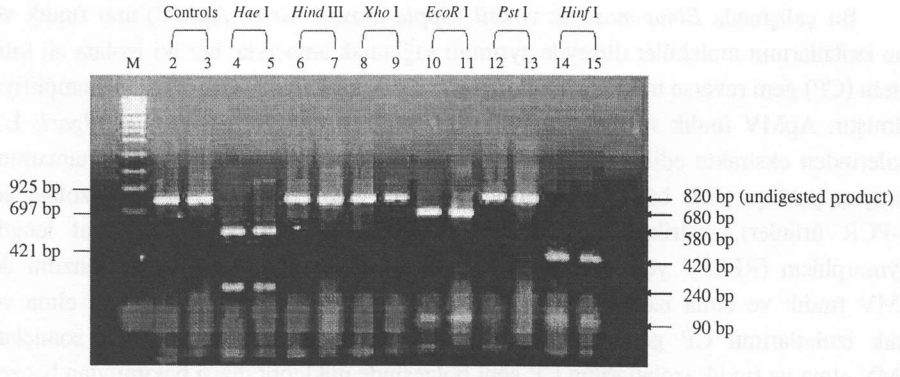

Figure 3. Restriction enzyme fragment length polymorphism (RFLP) analysis of the CP protein gene of ApMV hazelnut and apple isolates. M: Marker (Lambda-pUC mix marker, MBI Fermantas); Controls: undigested RT-PCR product of ApMV apple isolate (line 2) and hazelnut isolate (line 3); restriction enzyme treated RT-PCR products of ApMV apple isolate (lines 4, 6, 8, 10, 12, 14) and hazelnut isolate (5, 7, 9, 11, 13, 15). Arrows indicate the size of undigested and digested products.

mahaleb differs from that of the USA apple isolates [reviewed in (Petrzik and Lenz, 2002)]. The presence of several nucleotide insertions (6 to 15 nucleotides) was shown in some ApMV isolates (Petrzik and Lenz, 2002) which resembles to the features of some PNRSV isolates in which 6 nt insertion determined after nucleotide 123 [reviewed in (Petrzik and Lenz, 2002)]. These studies showed that the length of CP gene of ApMV is variable due to either deletions or insertions. These findings brought a question of that whether ApMV isolates from hazelnut and apple crops growing in Samsun and Tokat provinces, respectively, have molecular variability depending on host or region. Only limited number of isolates from two different hosts was used in the present study for RFLP analysis. Three enzymes (*Hae* I, *EcoR* I and *Hinf* I) digested the CP region of ApMV apple and hazelnut isolates and gave the same band patterns (Figure 3). So far, there is no data providing RFLP differention of ApMV isolates. Therefore, ten restriction enzymes used in this study were randomly chosen, but no any variability was obtained between apple and hazelnut isolates. Other enzymes in future experiments may differentiate ApMV isolates in the CP region. This preliminary result may suggest that hazelnut and apple isolate of ApMV have nucleotide homology in their CP gene. However, further studies are needed for characterization of ApMV isolates from different hosts and other parts of Turkey by RFLP and sequence analysis of the CP gene.

ÖZET

ELMA MOZAYİK VİRÜSÜ (*APPLE MOSAIC VIRUS* ;ApMV) ELMA VE FINDIK İZOLATLARININ MOLEKÜLER AYRIMINA YÖNELIK BİR ÖN ÇALIŞMA

Bu çalışmada *Elma mozayik virüsü* (*Apple mosaic virus*; ApMV)'nün fındık ve elma izolatlarının moleküler düzeyde ayrımını sağlamak amacıyla, her iki izolata ait kılıf protein (CP) geni reverse transkriptaz-polimeraz zincir reaksiyonu (RT-PCR) ile amplifiye edilmiştir. ApMV fındık ve elma izolatları ile enfekteli fasulye (*Phaseolus vulgaris* L.) bitkilerinden ekstrakte edilen toplam RNA'lar kullanılarak, ApMV CP genin tamamını kapsayan yaklaşık 820 baz çiftlik bir PCR ürünü elde edilmiştir. Her iki izolata ait RT-PCR ürünleri, restriksiyon enzimleri ile kesilerek 'Restriction fragment length polymorphism (RFLP)' yöntemi ile analiz edilmiştir. On farklı restriksiyon enzimi ile ApMV fındık ve elma izolatlarına ait PCR ürünlerinin kesilmesi sonucunda, elma ve fındık izolatlarının CP geni bölgesinde polymorfizim gözlenmemiştir. Bu sonuçlar, ApMV elma ve fındık izolatlarının CP geni bölgesinde nükleotit dizisi bakımından benzer olabileceklerini göstermiştir. Bununla birlikte, farklı konukçulardan ve bölgelerden elde edilecek ApMV izolatlarının CP geni bölgesinin daha detaylı araştırılmasına gereksinim duyulmaktadır.

Anahtar Kelimeler: ApMV, RT-PCR, RFLP, kılıf proteini, elma, fındık

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TLC Analysis of Some Pesticide Residues Using Enzyme Inhibition Techniques

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ABSTRACT

Thin layer chromatography (TLC) is used for semi-quantitative and qualitative analysis of wide variety of pesticides. It is particularly necessary for the nonvolatile or labile pesticides which can not be analysed with Gas Chromatography (GC). The objective of this study was to determine retention factor (R_f), relative retention factor (RR_f) and minimum detectable quantities (MDQ) of parathion-methyl, dichlorvos, oxamyl, chlorpyrifos, carbaryl and methomyl with "enzyme inhibition with cow liver extract and β-naphthyl-acetate substrate [EβNA] detection method" and "enzyme inhibition with horse blood serum and acethythiocoline iodide substrate (EAcI-horse) detection method". The MDQ's of the pesticides were verified in the presence of cleaned-up wheat-grain extract. The limit of detections (LOD) of the compounds were also calculated.

Key words: Enzyme inhibition, TLC-detection methods

INTRODUCTION

TLC has been used for many years in pesticide residue analysis due to its ability to resolve difficult separations and provide semi-quantitative information on pesticide levels in samples. The TLC detection may also be used together with the GC or High Pressure Liquid Chromatography (HPLC) detection for confirmation of the residues in other laboratories (Rathore and Sharma, 1992; Ambrus et al., 1996).

Separation and reproducible R_f values are interdependent in TLC. It was proven that, to ensure the reproducibility of the R_f values and the required efficiency, the conditions have to be controlled. There are a lot of parameters influencing reproducibility of HR_f (R_f x 100) such as, type and quality of the adsorbent, activity of the layer, layer thickness, saturation of the chamber, humidity of the air, temperature, and elution solvent. The use of the marker compounds have proven to be very satisfactory for this purpose (Lantos, 1998).

The MDQ's of the marker compounds result in well-defined spots only with optimum conditions. They should be spotted on each plate on which qualitative and semi-quantitative determinations are carried out. If the marker compounds are well detectable and their R_f values are within the expectable range the analyst can be sure, and can demonstrate it at the same time, that method was applied properly. The R_f values of the marker compounds can also be used as reference for the RR_f values which greatly facilitates the identification of the spots detected on the plates. The marker compounds selected should be stabile in standard solutions, be sensitive for the detection methods, have reproducible R_f values (Ambrus et al., 1981; Hargitai, 1984).

A marker compound was selected for each detection Füzesi et al. (1998) determined the MDQ and R_f values of marker compounds referring to the different detection methods.

In the previous work, the applicability of TLC in pesticide residue analysis was tested by using the following detection methods: *o*-tolidine plus KI, $AgNO_3$ plus UV radiation, photosynthesis inhibition, bioassay with fungi spores, cow liver enzyme inhibition, and horse blood serum enzyme inhibition (Füzesi et al., 1998). Enzymatic reactions can be monitored on a TLC plate, and the end products can be detected. Various pesticides inactivate or inhibit enzymes associated with the animal nervous tissue. Enzyme inhibition detection methods are specific for enzyme inhibiting compounds-especially phosphoric and thio-phosphoric acid esters and carbamate pesticides. Plant extracts usually do not interfere with the enzymatic detection (Ambrus et al., 1996; Lantos, 1998).

The maximum residue limits of pesticides, tested in this study, reported according to Türk Gıda Kodeksi, FAO Codex, and EU limits are given in Table 1.

This study was undertaken to investigate the possibilities of applying cow liver enzyme EßNA and EAcI-horse TLC detection method, providing an alternative cost effective analytical procedure for pesticide residues in plant commodities using grain commodity.

Table 1. Maximum residue limits (MRL) of tested pesticides in grain (mg kg^{-1}).

Compound	National[a]	FAO[b]	EU[c]
Carbaryl	0.5	2	0.5
Chlorpyrifos	0.05	0.5	0.05
Dichlorvos	1	1[d]	2
Methomyl		0.5	0.05
Oxamyl	-	-	-
Parathion-M	0.2	-	-

[a] Türk Gıda Kodeksi. http://www.kkgm.gov.tr/Genel/index.asp? Prm=/Mevzuat/ Kodeks.asp? Adres=Kodeks List.htm (Updated 2003).

[b] FAO Codex, [c] EU limits http://europa.eu.int/comm/food/plant/protection/resources/mrl_commodity.pdf,

[d] wheat flour

MATERIALS and METHODS

Materials

Equipment

General laboratory equipment and TLC basic set were used in this study. Gel Permation Chromatography (GPC) apparatus were used for cleaning-up wheat extracts.

Pesticides

Parathion-methyl, dichlorvos and oxamyl were used as marker compounds, and chlorpyrifos and carbaryl as selected compounds in the EβNA detection methods. Parathion-methyl, methomyl and oxamyl were used as marker compounds in the EAcI-horse detection methods (Ambrus et al., 1981 and 1996). The standard of active ingredients was obtained from Dr. Ehrenstorfer Laboratories GmbH, Germany.

Chemicals

All chemicals used were analytical grade and solvents were reagent grade (Merck).

Reagents

The following reagent solutions described, by Ambrus et al. (1981 and 1996), were used for both enzym inhibition techniques.

a) Enzyme solution.

b) β-naphthyl-acetate: 1.25 mg ml^{-1} solutions in ethanol.

c) Echtblau-salt (Fast Blue BB salt): 10 mg salt in 16 ml bi-distilled water.

d) Horse blood serum.

e) 0.05 mol tris-buffer: 3.04 g tris aminomethane in 500 ml bidistilled water

f) Acetyl thiocoline iodide: 1.5 mg ml^{-1} water solution.

g) 2,6-dichlorophenol-indophenol Na-salt: 0.5 mg ml^{-1} solution in distilled water

h) Silica gel: Precoated silica gel plates (Merck: 1.05721).

Methods

Elution of pesticides on TLC plates

The method of elution on TLC layer is described by Ambrus et al. (1996) and Tiryaki et al. (2003) The mixture of marker and/or selected compounds were spotted on each plate in different MDQ's (Table 2, Table 3 and Table 4). Rf values were determined in developing tanks which was kept in water bath thermostated at 23°C in order to reduce the effect of temperature variation in the laboratory. The eluent (ethylacetate, EtAc) was equilibrated with the vapour phase by inserting filter paper in the developing tank and

waiting for minimum 30 minutes before the plates were put into the tanks. The plate was placed entire in 1 cm eluting solvent. The eluent was allowed to run up to 11 cm from the start line (Ambrus et al., 1996).

EβNA detection method

The extract and/or standard solutions were chromatographed on Silica gel 60 plate. After elution, the plate was air dryed and treated it with bromine vapour.

Treatment with bromine: 25 ml baker was placed into the bottom of the developing tank. Using a safety pipette 0.5-1 ml bromine was transferred into the baker. The tank covered and waited for few minutes until the bromine vapour saturated the tank. The dry plate was placed into the tank for 15 minutes and removed. It was kept for about 45 min in a well ventilated fume hood to remove the excess bromine.

Preparation of the enzyme solution is given below: The fresh liver of cattle was cut into small pieces. 10 g portion were weighed into 90 ml bi-distilled water and homogenised with Ultra Turrax or high speed blender. The homogenate was centrifugated at 4000 rpm for 10 minutes and the supernatant were collected in 10 or 20 ml portion. Then they were placed into deep-freezer until use. The enzyme concentrate was diluted 3 times with bi-distilled water before use.

Enzyme solution was sprayed the plate until it gets thoroughly wet. Afterwards it was placed it into an incubator or oven at 37°C for 30 minutes. Instead of an incubator, simple way was introduced by Asi[1] ; which is: a developing tank with ground glass lid can be very efficiently used. The tank was saturated with water in an oven at 37°C and kept about 2 cm water in the tank. The upper 3 cm of the adsorbent layer was removed from the plate, then the cleaned part of the plate was inserted in the water and the lid was closed. The tank was kept for 30 min in the oven. The excess water was removed with air stream after incubation. The substrate solution (mixture of 10 ml β-naphthyl-acetate solution and 16 ml Echtblau-salt solution) was sprayed to the plate. Colour reaction was observed with the white spots occur in pink background (Ambrus et al., 1996).

EAcI-horse detection method

The extract and/or standard solutions were chromatographed on Silica gel 60 plate. After elution, the plate was air dryed and treated with bromine vapour.

Horse blood enzyme solution was prepared accordingly: The clot (coagulated blood) was brake with a glass rod, transferred it into centrifuge tubes and centrifugated at

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4000 rpm for 10 minutes. Serum was collected in 10 ml portions and stored in deep-freezer until use. The cholinesterase activity was determined by Ellman method (Ellman et al., 1961) and serum was diluted with tris-buffer before use to obtain about 570 U l^{-1} activity of horse serum. If Ellman test cannot be carried out the enzyme activity can be determined experimentally to obtain the best sensitivity.

Horse blood serum was sprayed the plate as in EßNA method. After incubation at 37°C for 30 minutes, excess of water was removed with air stream. The substrate solution (acetyl thiocoline iodide in 1.5 mg ml^{-1} water solution) was sprayed to the plate and incubated again for 15 min. The excess water was removed with air stream and sprayed the plate with the solution of 2,6-dichlorophenol-indophenol Na-salt (0.5 mg ml^{-1} solution in distilled water). Colour reaction was observed with blue spots in white background (Ambrus et al., 1996).

Extraction and clean-up

GPC column (200 mm x 10 mm glass column) was filled with 8 g SX-3 gel as described in the manual (Anonymous,1998). Calibration of the GPC column, extraction and clean up were carried out as described by Füzesi et al. (1998).

RESULTS and DISCUSSION

Marker compounds results

The retention factors (R_f) were measured with 3 runs for each compound under the specific conditions described for EβNA and EAcI-horse detection methods. The spotting detail for determining the R_f, and RR_f values of EβNA-marker compounds, EβNA-selected compounds and EAcI-horse-marker compounds are given in Table 2, Table 3 and Table 4, respectively.

The R_f, RR_f, and their co efficient of variations (CV's) and MDQ's determined with EβNA and EAcI-horse detection methods are shown in Table 5. To compare our data Füzesi et al. (1998)'s included in Table 5. Our findings on the R_f and RR_f values were in close agreement with the reported ones, except carbaryl and oxamyl. May be the main reason of the situation is the working conditions in the our laboratory. A lot of factors influencing the reproducibility of R_f is explained by Lantos, 1998. To ensure correct result, marker compounds, and selected compounds and sample extract must be spotted on TLC plate and each plate must be evaluate individually. It is important to eliminate the effect of working conditions on R_f.

As to MDQ results, our findings are lower than Füzesi et al. (1998)'s findings, except oxamyl with the 2 ng and 10 ng in EβNA and EAcI-horse detection, respectively (Table 5).

Table 2. The spotting format and determined R_f, RR_f and MDQ's of the marker ompounds with the EβNA detection method (Average of 3 plate).

Spot No.	Applied MDQ eq.[a]	R_f of Dichlorvos	R_f of Oxamyl	R_f of Parathion-M (Rf_{ref}[d])	RR_f of Dichlorvos	RR_f of Oxamyl
1	5	0.498	0.154	0.722	0.690	0.213
2	2	0.497	0.154	0.717	0.693	0.215
3	1.5	0.498	0.157	0.716	0.696	0.219
4	1	0.498	0.159	0.719	0.693	0.221
5	0.75	0.497	nd[c]	0.714	0.696	nd
6	0.5	0.489[b]	nd	0.718	0.681	nd
7	0.5	0.498	nd	0.724	0.689	nd
8	0.75	0.500	nd	0.725	0.690	nd
9	1	0.500	0.162	0.722	0.692	0.224
10	1.5	0.500	0.156	0.725	0.690	0.215
11	2	0.498	0.156	0.727	0.686	0.214
Average R_f		0.498	0.156	0.721	0.690	0.217
Standard deviation		0.003	0.003	0.004	0.004	0.004
Variance		1.01E-05	7.43E-04	1.7E-05	1.92E-05	1.63E-05
Coefficient of variation		0.006	0.017	0.006	0.006	0.019

[a] MDQ is the value reported in the background technical document by Füzesi et al. (1998). 1 MDQ for parathion-m, dichlorvos, and oxamyl are 1 ng, 20 ng and 10 ng, respectively, in the EβNA detection method.

[b] The R_f values of the spot of the lowest detectable amount of materials are underlined.

[c] Not detected

[d] Rf_{ref} =R_f values of reference marker compound (parathion-m)

Table 3. The spotting format and determined R_f, RR_f and MDQ's of the selected compounds with the EβNA detection method (Average of 3 plate).

Spot No.	Applied MDQ eq.[a]	R_f of Carbaryl	R_f of Chlorpyrifosl	R_f of Parathion-M (Rf_{ref}[c])	RR_f of Carbaryl	RR_f of Chlorpyrifos
1	5DQ	0.631	0.742	-	0.866	1.019
2	2DQ	0.630	0.736	-	0.864	1.010
3	2mDQ	-	-	0.720	-	-
4	1.5DQ	0.630	0.741		0.864	1.017
5	1DQ	0.637	0.735		0.874	1.008
6	1mDQ	-	-	0.729	-	-
7	1DQ	0.639	0.737		0.877	1.011
8	0.75DQ	0.649	0.741		0.891	1.017
9	0.75mDQ	-	-	0.730	-	-
10	0.5DQ	0.652[b]	0.744		0.895	1.021
11	1.5mDQ	-	-	0.736	-	-
Average R_f		0.638	0.739	0.729	0.876	1.015
Standard deviation		0.009	0.003	0.007	0.013	0.005
Variance		8.8E-05	1.2E-05	4.8E-05	0.00017	2.2E-05
Coefficient of variation		0.015	0.005	0.010	0.015	0.005

[a] MDQ is the value reported in the background technical document by Füzesi et al. (1998). 1 DQ (selected copmpounds) for carbaryl, and chlorpyrifos are 10 ng, 0,5 ng, respectively. 1 mDQ of reference compond (parathion-m) is 1 ng in the EβNA detection method.

[b] The R_f values of the spot of the lowest detectable amount of materials are underlined.

[c] Rf_{ref} =R_f values of reference marker compound (parathion-m)

Table 4. The spotting format and determined R_f, RR_f and MDQ's of the selected compounds with the EAcI-horse detection method (Average of 3 plate).

Spot No.	Applied MDQ eq.[a]	R_f of Oxamyl	R_f of Methomyl	R_f of Parathion-M (Rf_{ref}[d])	RR_f of Oxamyl	RR_f of Methomyl
1	5	0.130	0.320	0.672	0.193	0.476
2	2	0.132	0.323	0.682	0.193	0.473
3	1.5	0.129	0.321	0.677	0.190	0.475
4	1	0.130[b]	0.321	0.677	0.193	0.475
5	0.75	ndc	0.323	0.685	nd	0.471
6	0.5	nd	0.324	0.688	nd	0.471
7	0.5	nd	0.326	0.689	nd	0.472
8	0.75	nd	0.321	0.685	nd	0.469
9	1	0.130	0.321	0.682	0.191	0.471
10	1.5	0.129	0.321	0.680	0.189	0.472
11	2	0.125	0.320	0.682	0.183	0.469
Average R_f		0.129	0.322	0.682	0.190	0.473
Standard deviation		0.002	0.002	0.005	0.004	0.002
Variance		5.43E-06	2.8041E-06	2.67E-05	1.37E-05	5.3597E-06
Coefficient of variation		0.018	0.005	0.008	0.019	0.005

[a] MDQ is the value reported in the background technical document by Füzesi et al. (1998). 1 MDQ for parathion-m, methomyl, and oxamyl are 2 ng, 10 ng and 2 ng , respectively in the EAcI-horse detection method.

[b] The R_f values of the spot of the lowest detectable amount of materials are underlined.

[c] Not detected

[d] Rf_{ref} =R_f values of reference marker compound (parathion-m)

Table 5. Summary of R_f, RR_f and MDQ's of the compound determined with the EβNA and EAcI-horse detection method.

Compound	Reproducibility of R_f values[a]					Reproducibility of RR_f[b] values		Reported[c]		Found	
	1	2	3	Aver	CV %	Aver	CV%	R_f	RR_f	MDQ	MDQ
EβNA											
Parathion-M	0.752	0.702	0.708	0.721	3.81	1	0	0.669	1	1	0.5
Dichlorvos	0.520	0.487	0.488	0.498	3.83	0.691	0.34	0.505	0.755	20	10
Oxamyl	0.158	0.159	0.152	0.156	2.46	0.217	4.22	0.189	0.283	10	10
Carbaryl	0.632	0.634	0.649	0.638	1.43	0.831	7.75	0.598	0.894	10	5
Chlorpyrifos	0.736	0.734	0.748	0.739	1.11	1.011	0.20	0.669	1	0.5	0.25
EAcI-horse											
Parathion-M	0.680	0.66	0.703	0.681	3.12	1	0	0.669	1	2	1
Oxamyl	0.124	0.128	0.134	0.129	3.71	0.189	3.08	0.189	0.283	2	2
Methomyl	0.320	0.329	0.314	0.321	2.30	0.472	5.42	0.363	0.543	10	5

[a] EtAc –precoated silica gel system

[b] RR_f =R_f / R_f of reference compound parathion-m

[c] Reported R_f and MDQ by Füzesi et al. (1998).

Verification of MDQ of the pesticides in the presence of cleaned up wheat extract

The objective of this study is to test the detectability of compounds in the presence of co-extractives being in the cleaned-up extracts. If the co-extractives affect the detectability of the compounds then the sample equivalent spotted onto the TLC plates should be reduced or further cleanup may be necessary to achieve the required sensitivity.

The verification tests were performed in three replicates with the blank extracts of wheat after the GPC clean-up. The cleaned extract was applied on the TLC plate and spotted over it with MDQ (in solvent). The amount of extract (sample equivalent in per volume) that could be spotted on to the TLC plate were found experimentally. The details of the chromatogram and spotting format for verifying the MDQ values with the EβNA detection method for both marker and selected compounds, and EAcI-horse detection method for marker compounds are given in Table 6, Table 7 and Table 8, respectively. As it can be seen in these three tables, 10 mg sample equivalent applied in 20 µl did not affect the detectability of the compounds with the enzyme inhibition procedures. The limit of detection (LOD) was calculated according to equation (1).

$$LOD = MDQ\ (ng)/M\ (mg) \qquad (1)$$

where M is the sample equivalent applied on the layer.

Table 6. The verification data of MDQ's of the marker compounds with the EβNA detection method in the presence of wheat extract (average of 3 plate).

Spot No.	Applied MDQ eq.[a]	R_f of Oxamyl	R_f of Dichlorvos	R_f of Parathion-M (Rf_{ref}[d])	RR_f of Oxamyl	RR_f of Dichlorvos
1	E[b]	-	-	-	-	-
2	E + MDQ	0.162[c]	0.288	0.661	0.715	0.436
3	E+1.5MDQ	0.175	0.291	0.668	0.709	0.435
4	E+ 2MDQ	0.167	0.294	0.668	0.692	0.439
5	E+ 3MDQ	0.169	0.297	0.664	0.718	0.447
6	MDQ in solv.	0.164	0.292	0.664	0.764	0.440
Average R_f		0.167	0.489	0.707	0.237	0.691
Standard deviation		0.005	0.001	0.002	0.006	0.003
Variance		2.39E-05	1.52E-06	6.29E-06	3.9E-05	1.2665E-05
Coefficient of variation		0.029	0.002	0.003	0.026	0.005

[a] Found MDQ values in the laboratory; 1 MDQ for parathion-m, dichlorvos, and oxamyl are 0.5 ng, 10 ng and 10 ng, respectively, in the EßNA detection method (Table 2)

[b] Wheat sample extract; 10 mg sample equivalent in 20 µl

[c] The R_f values of the spot of the lowest detectable amount of materials are underlined.

[d] $Rf_{ref} = R_f$ values of reference marker compound (parathion-m)

Table 7. The verification data of MDQ values of the selected compounds with the EβNA detection method in the presence of wheat extract (average of 3 plate).

Spot No.	Applied MDQ eq.[a]	R_f of Carbaryl	R_f of Chlorpyrifos	R_f of Parathion-M (Rf_{ref}[d])	RR_f of Carbaryl	RR_f of Chlorpyrifos
1	E[b]	-	-	-	-	-
2	E+ DQ	0.621	0.711	-	0.886	1.016
3	E+ 1.5DQ	0.621	0.711	-	0.886	1.016
4	E+ 2DQ	0.622	0.711	-	0.888	1.016
5	E+ 3DQ	0.620	0.711	-	0.886	1.016
6	mDQ in solv.			0.700		
Average R_f		0.621	0.711	-	0.887	1.016
Standard deviation		0.001	0.0002	-	0.001	0.0002
Variance		5.2E-07	2E-08	-	1E-06	5E-08
Coefficient of variation:		0.00116	0.0002	-	0.0012	0.0002

[a] Found MDQ values in the laboratory; 1 DQ (selected copmpounds) for carbaryl,and chlorpyrifos are 5 ng, and 0.25 ng, respectively. 1 mDQ of reference compond (parathion-m) is 0.5 ng in the EβNA detection method (Table 3).

[b] Wheat sample extract ; 10 mg sample equivalent in 20 µl.

[c] The R_f values of the spot of the lowest detectable amount of materials are underlined.

[d] $Rf_{ref} = R_f$ values of reference marker compound (parathion-m).

Table 8. The verification data of MDQ values of the marker compounds with the EAcI-horse detection method in the presence of wheat extract (average of 3 plate).

Spot No.	Applied MDQ eq.[a]	R_f of Oxamyl	R_f of Methomyl	R_f of Parathion-M (Rf_{ref}[d])	RR_f of Oxamyl	RR_f of Methomyl
1	E[b]	-	-	-	-	-
2	E + MDQ	0.144	0.328	0.659	0.219	0.498
3	E+1.5MDQ	0.142	0.328	0.666	0.213	0.492
4	E+ 2MDQ	0.135	0.328	0.668	0.203	0.491
5	E+ 3MDQ	0.135	0.334	0.669	0.202	0.499
6	MDQ in solv.	0.143	0.335	0.705	0.202	0.476
Average R_f		0.140	0.331	0.673	0.208	0.491
Standard deviation		0.004	0.004	0.018	0.008	0.009
Variance		1.738E-05	1.34E-05	0.0003	5.73E-05	8.63E-05
Coefficient of variation		0.030	0.011	0.027	0.036	0.019

[a] Found MDQ values in the laboratory; 1 MDQ for parathion-m, oxamyl, and methomyl are 1 ng, 2 ng and 5 ng, respectively, in the EacI-horse detection method (Table 4).

[b] Wheat sample extract; 10 mg sample equivalent in 20 µl.

[c] The R_f values of the spot of the lowest detectable amount of materials are underlined.

[d] $Rf_{ref} = R_f$ values of reference marker compound (parathion-m).

The limit of detection of the tested pesticides are summarised in Table 9. The LOD levels of any detection methods should not be higher than MRL (LOD ≤ MRL). As it is showed in Table 1 and Table 9, detection limit of the tested pesticides are lower/equal than their MRLs, except oxamyl.

Table 9. Verification of the MDQ of pesticides in the wheat extracts and their limit of detections (LOD).

TLC Detection Method		Active Ingredients	Found MDQ ng	LOD[a], mg kg^{-1}
	Marker	Parathion-M	0.5	0.05
		Dichlorvos	10	1
		Oxamyl	10	1
		Carbaryl	5	0.5
EβNA	Selected	Chlorpyrifos	0.25	0.025
		Parathion-M	1	0.1
EAcI-horse	Marker	Oxamyl	2	0.2
		Methomyl	5	0.5

[a] LOD=MDQ (ng)/10 mg sample equivalent; ng mg^{-1} ; mg kg^{-1}

The results of this study have demonstrated that EβNA detection method is suitable for the analysis of carbaryl, dichlorvos, chlorpyrifos and parathion-m within the MRL limit. Similarly, EAcI-horse detection method is also suitable for the analysis of methomyl, and parathion-m.

ÖZET

BAZI PESTİSİT KALINTILARININ ENZİM ENGELLEME METODU KULLANILARAK İNCE TABAKA KROMATOGRAFİSİ İLE ANALİZ EDİLMESİ

İnce tabaka kromatografisi (TLC), özellikle GC'de saptanamayan, pek çok pestisitin, kalitatif ve yarı kantitatif analizi için uygun bir tekniktir. Bu çalışmada bazı pestisitlerin (parathion-methyl, dichlorvos, oxamyl, chlorpyrifos, carbaryl ve methomyl) 'karaciğer ekstraktı ve β-naphthyl-acetate substratı enzim engelleme metodu (EβNA) ve 'at kanı serumu ve acethythiocoline iodide substratı enzim engelleme metodu (EAcI-horse)' kullanarak TLC ile alıkonma faktörleri (R_f), relatif alıkonma faktörleri (RR_f) ve en az belirlenebilen miktarları (MDQ) bulunmuştur. Temizlenmiş (clean-up) buğday ekstraktları tabakaya uygulanmış, üzerine solvent içindeki MDQ'lar spotlanarak bulunan MDQ değerleri doğrulanmıştır. Bu pestisitlerin saptama limitleri de (LOD) ayrıca hesaplanmıştır. Oxamyl haricinde, test edilen pestisitlerin enzim engelleme metodları ile MRL seviyelerinin altında analiz edilebileceği belirlenmiştir.

Anahtar Kelimeler: Enzim engelleme metodu, TLC saptama metotları

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Detection of Potato Leafroll Virus Infection from Dormant Potato Tubers, Leaf Tissues and Green Peach Aphid (*Myzus percicae* Sulzer) by RT-PCR

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ABSTRACT

A reverse transcription polymerase chain reaction (RT-PCR) was used for the detection of PLRV from potato leaves, dormant potato tubers belonging to Marfona (60), Agria (60), Granola (60), the green peach aphid (*Myzus percicae* Sulzer) feeding on PLRV-infected potato plants in a greenhouse and winged aphids caught in yellow water traps in the potato fields of in Central District of Erzurum. For this purpose, a total of 180 dormant tubers were tested in order to detect PLRV-infected tubers by RT-PCR and then, whether PLRV-infected or not, all tubers were planted in pots and grown in a greenhouse to confirm the presence of PLRV. When dormant tubers were tested by RT-PCR, it was found that 14 of 60 tubers for Marfona, 18 of 60 tubers for Agria and 22 of 60 tubers for Granola were found to be infected with PLRV. However, when leaf tissues were used for test, in this case, it was determined to be infected with PLRV of 16 of 60 tubers for Marfona, 22 of 60 tubers for Agria, 28 of 60 tubers for Granola. When individual or a group of aphids (50 aphids) (*Myzus persicae* Sulzer) collected from PLRV-infected potato plants in a greenhouse were tested by RT-PCR, PLRV was detected from both individual and fifty aphids. However, PLRV was not detected from individual or fifty aphids caught from yellow water traps in potato fields in spite of nested PCR.

Key words: PLRV, detection, dormant tubers, leaves, aphids, RT-PCR

INTRODUCTION

The transmission of diseases agents from a host to another is the most important key in their biology and most of plant virus diseases are transmitted by a variety of vectors. However, the relationship between viruses and their vectors is highly complex and dependent on host, inoculum, and favorable environment. The most important vectors carrying plant viruses belonging to Homoptera (aphids, hoppers, whiteflies, and meal bugs) order and aphids are responsible for transmission of 66% of the 370 plant viruses with invertebrate vectors (Mathews, 1992). One of the most efficient vector of potato viruses is the green peach aphid (*Myzus percicae Sulzer*) (Sylvester, 1980; Peters and Jones, 1981; Singh et al., 1997). Although there are number of viral pathogens that

can impact seed potato stocks worldwide (Salazar, 1996), the most common viruses causing major economic losses in seed potato crops single or in combination in Turkey are Potato leafroll virus (PLRV, a *Polerovirus*); *Potato virus Y* (PVY, *Potyvirus*) and *Potato virus S* (PVS, *Carlavirus*) (Bostan and Haliloğlu, 2004). Of these viruses; PLRV is transmitted in a nonpropagative, circulative manner by several aphid species and *Myzus persicae* (Sulzer) is generally considered the most efficient, cosmopolitan and commonly abundant vector of PLRV (Peters and Jones, 1981; Woodford et al., 1995; Robert and Bourdin, 2001). Acquisition and transmission of PLRV by aphids is a prolonged process, requiring styled penetration to the phloem, followed by an incubation period of a day or so and then another phloem penetration to transmit the virus (Bagnall, 1988). Because PLRV persists throughout the aphid's life, it can be spread over longer distances by wind-borne winged aphids (Peters and Jones, 1981). On the other hand, PLRV is confined to phloem tissue of infected plants and occurs in low titers in plant tissues and its vectors (Peters and Jones, 1981). Therefore, to study virus-vector relationships, a highly sensitive method for detecting of the viruses is required.

The polymerase chain reaction (PCR) is an extremely sensitive and specific technique (Saiki et al., 1988; Henson and French, 1993), and reverse transcription PCR (RT-PCR) has been used to detect PLRV from leaves and dormant tubers (Barker et al., 1993; Singh and Singh, 1996; Singh et al., 1996; Singh, 1998, 1999, Nie and Singh, 2001) and aphids (López-Moya et al., 1992; Hadidi et al., 1993; Singh et al., 1995; 1996, 1997; Singh, 1999).

The objective of this study was to determine of PLRV from dormant tubers, potato leaves, individual or a group of *Myzus percicae* Sulzer feeding on PLRV-infected potato plants and aphids caught in yellow water traps in potato fields by RT-PCR.

MATERIALS and METHODS

Potato cultivars and aphid vectors

A total of 180 tubers of potato cvs Marfona (60), Agria (60) and Granola (60) (obtained from Agricultural Research Institute of East Anatolia, Erzurum, Turkey) were tested in order to determine of PLRV-infected tubers by RT-PCR. After broken dormancy (100 days storage at 4°C), tubers were planted into pots and grown in a greenhouse. In order to confirm the presence of PLRV, leaf tissues collected from these plants were retested by RT-PCR and about 100 of green peach aphid (*Myzus persicae* Sulzer) were placed on PLRV-infected plants. Aphids were collected from PLRV-infected plants one month later and stored in 70 % ethanol at room temperature until to be used for RNA extraction. On the other hand, in order to determine aphids caught in five yellow water traps from two potato fields in Central District of Erzurum during August of 2004 and 2005 were also tested by RT-PCR.

RNA extraction from dormant tubers and leaves

The nucleic acid extraction from dormant tubers, leaves and aphids was carried out as described by previously Singh et al. (2002). Briefly, the extraction buffer 300 µl of 0.1 M Tris HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.65 % Na2SO3 and containing 10 U of RNase-free DNase I (Roshe Molecular Biochemicals, Laval, Oue., Canada) was combined with 6 drops (150-180 µl) of sap, obtained by Tuber Slicer (Electrowerk, Behcke and Co., Hannover, Germany). Samples were vortexed for 10-15 s and incubated at 37°C for 10 min. Total RNA was extracted once using water-saturated phenol and once with chloroform:isoamyl alcohol (24:1) and then precipitated from the aqueous phase with 1 vol. of isopropenol in the presence of 0.3 M sodium acetate (-20°C, over night). The precipitate was collected by centrifugation (12 000 g, 15 min, 4°C), washed with 70% ethanol, dried under vacuum and dissolved in 1000 µl (leaves) 100 µl (tubers) of sterile water.

RNA extraction from aphids

For the extraction of RNA from individual or fifty aphids collected from PLRV-infected plants in a greenhouse, from healthy potato plants and potato field were placed in 1.5 ml eppendorf tubes and dried in speed vacuum for 10 min. For this purpose, dried aphids were ground with a Pellet Pestle Motor and disposable polypropylene pestles. After initial homogenization, 25 µl of DNase extraction buffer (same as leaves and tubers without Na_2SO_3) was added and samples were further mixed. The samples were wortexed for 10 second and incubated for 10 min. SDS was added to 1% final concentration and Proteinase K to 0.5 mg/ml and then incubated for 10 min at 65°C. Samples were extracted once using phenol saturated with water and once with chloroform:isoamyl alcohol (24:1). RNA was precipitated by incubating overnight at -20°C in the presence of 2.5 volumes of ethanol, 0.3 M sodium acetate and 20 µg glycogen. After samples were centrifuged at 12 000 g, 15 min, 4°C, washed with 70% ethanol, dried under vacuum and dissolved in 10 µl of sterile water.

Reverse transcription polymerase chain reaction

For the reverse transcription reaction, 2.5 µl of RNA extracted from tubers, leaves and aphids were incubated at 65°C for 8 min and then chilled on ice for 5 min to denature the RNA. The reverse transcription mixture (7.5 µl) was added to provide a final concentration of 20 ng/µl of reverse primer, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM DDT, 2.5 mM $MgCl_2$, 2.5 mM of each dNTPs [dATP, dCTP, dGTP, dTTP, (Promega)], 20 U RNasin (Promega, Madison, WI) and 200 U Moleney Murine Leukemia virus-reverse transcriptase (MMLV-RT) (Invitrogen). Samples were incubated 1 h at 42°C for RT and subsequently incubated at 95°C for 3 min to terminate the reaction.

The forward primer (5'-CGC GCT AAC AGA GTT CAG CC-3') homologous to nucleotide (nt) 3670-3689 and the reverse primer (5'-GCA ATG GGG GTC CAA CTC

AT-3'), complementary to nt 4005-3986, for PLRV were used (Singh et al., 1995; Singh et al., 1997; Singh, 1999; Nie and Singh, 2001). PCR was performed using 2 µl of aliquots of the cDNA mixture in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM DDT, 1.5 mM MgCl2, 2.0 mM of each dNTPs, 0.1 µg each of antisense and sense primers, and 0.625 U of Taq polymerase (Sigma). Samples were amplified in 35 cycles with a denaturation step 94°C, primer annealing 60°C, primer extension 72°C (each step of 1 min duration) and final extension at 72°C (10 min). A total of 10 µl (leaves and tubers) and 15 µl (aphids) amplification products were electrophoresed on a 2.0% agarose gel containing 0.2 µg/ml ethidium bromide and photographed under UV illumination. A low mass ladder (Invitrogen) was used in the gel to determine the size of the amplified product.

RESULTS and DISCUSSION

Detection of PLRV from Dormant Tubers and Leaves

When test performed from dormant tubers, 14 of 60 for Marfona, 18 of 60 for Agria, and 22 of 60 for Granola were found to be infected with PLRV. However, when test carried out from leaf samples collected from greenhouse plants, in this case, the number of PLRV-infected samples were 16 of 60 for Marfona, 22 of 60 for Agria and 28 of 60 for Granola, as shown Table 1. This difference between the tests carried out from tubers and leaves may result from either the concentration of PLRV in some tubers was low or virus was spread from infected plants to healthy by aphids in the greenhouse. However, when nested PCR was performed from some samples giving weak bands on the first RT-PCR product obtained from tubers, bands were more intense and visible on photograph (Fig 1). This finding supports that the concentration of PLRV in some dormant tubers might be low owing to secondary infection. When total RNAs obtained from tubers were diluted up to 1:64, and then subjected to RT-PCR analysis, PLRV was visible on the gel up to dilution of 1:8 in Marfona, up to 1:16 in Agria and Granola. Repeat tests using five tubers from each cultivar gave similar results. The result of RT-PCR showed that PLRV can be detected reliable up to dilution of 1:16 in Agria and Granola, but up to only 1:8 in Marfona. On the other hand, the negative effect of nucleic acid dilution on RT-PCR product shows that the low concentration of polyphenollics and polysaccharides, which are known to inhibit PCR reaction (John, 1992; Hänni et al., 1995, Singh and Singh, 1996).

Table 1. Result of tests performed from dormant tubers and leaves by RT-PCR.

Cultivar	Number of tested tubers	Number of positive tubers	Number of positive leaves
Morfona	60	14	16
Agria	60	18	22
Granola	60	22	28

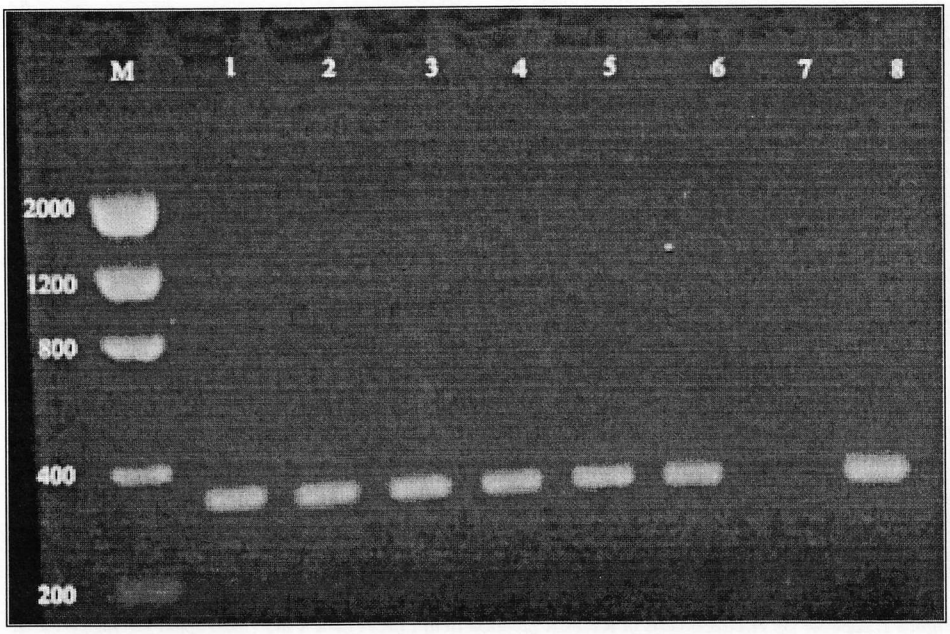

Figure 1. Agarose gel electrophoresis of RT-PCR products from PLRV infected dormant potato tubers and leaves. Lanes 1, 2 and 3: PLRV-infected field-grown dormant tubers; Lane 4, 5, and 6: leaf samples collected from PLRV-infected potato plants cvs Morfona, Agria and Granola; Lane 7: negative control (from healthy tuber); lane 8: positive control (from PLRV-infected potato plants); M, molecular size markers.

Detection of PLRV from aphids

When individual or a group of 50 aphids (*Myzus persicae*) collected from PLRV-infected potato plants in the greenhouse were tested by RT-PCR, PLRV was detected from both single and 50 aphids (Fig 2). However, PLRV was not detected from individual or 50 aphids caught in yellow water traps in potato field, in spite of the second test carried out using the first PCR product. The reason why aphids species caught from traps may be relatively inefficient vector of PLRV, these aphids species could be non-colonizing aphids on potato, PLRV carrying aphids might be mixed with non-viruliferous aphids or some aphid species caught in traps might include some subsidence inhibit RT-PCR. PLRV is transmitted by aphids colonizing on potato crops but not by all colonizing aphid species (Harrewijn, et al., 1981). As a matter of fact, *M. persicae*, *Aphis nasturtii* (buckthorn), *Aulacothum solani* (foxglove), *Macrosiphum euphorbiae* (potato), *Myzus ascolonicus* (shallot) and *Rhopalosiphoninus latysiphon* (bulb and potato) are colonizing on potato, only *M. persicae* is an efficient vector of PLRV (MacGillivray, 1981). However, PLRV is transmitted by *A. nasturtii* and *M. euphorbiae* and can be detected from these vectors by RT-PCR, but it was reported that

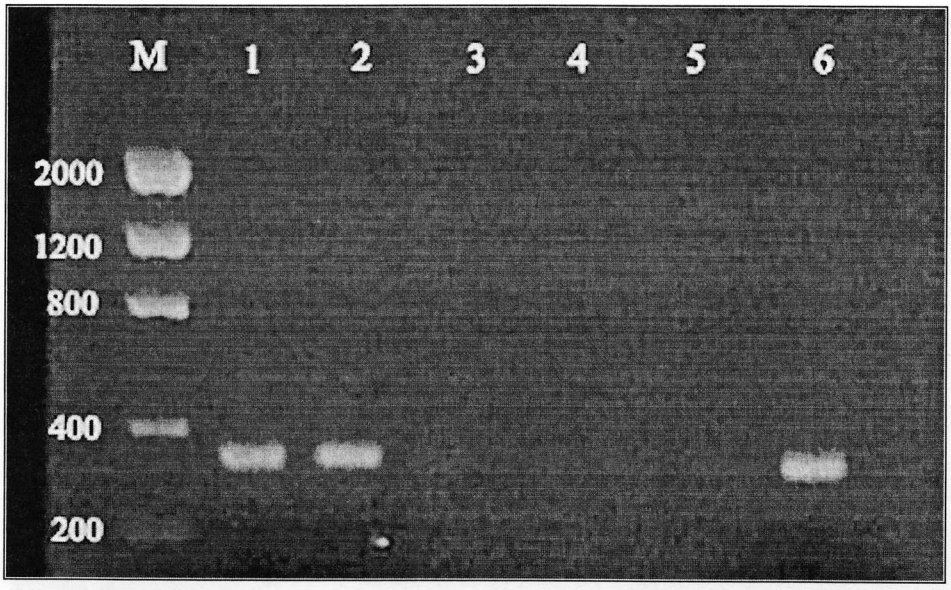

Figure 2. Agarose gel electrophoresis of RT-PCR products from PLRV. Lane 1 individual aphid (*Myzus percicae*; lane 2: a group of *Myzus percicae* collected from PLRV infected potato plants in a greenhouse; lanes 3 and 4: a group of aphids caught from yellow traps in potato field; lane 5: negative control (from healthy tuber); lane 6: positive control (from PLRV-infected potato plants); M, molecular size markers.

the efficiency of these vectors are poor (MacKinnon 1969; Tamada et al., 1984; Singh et al., 1996). On the other hand, transmission efficiency of potato viruses transmitted by aphids differ greatly based on aphid species, aphid biotypes, inoculum sources and host resistance to virus and vectors (Harrington et al., 1986; De Bokx and Piron, 1990; Wilson, and Jones, 1993; Novy et al., 2002). Within-field spread of PLRV is often by apterae walking from plant to plant (Hanafi et al., 1981; Flanders et al., 1991; Hodgson, 1991), and apterous *M. persicae* tents to be more efficient vector of PLRV than winged morphs, and nymphs are more efficient than adults (Robert, 1971; Ragsdale et al., 1994).

As a result, this study showed that the primer pairs can be used for the detection of PLRV from potato leaf, dormant tubers and viruliferous aphid *Myzus persicae* in Turkey. Furthermore, the same primer pairs can also be used for certification of PLRV. On the other hand, if nucleic acid extracted from dormant tubers and from leaf tissues will be used directly for the detection of PLRV by RT-PCR, in this case, no need to dilute of total RNA for these cultivars.

ÖZET

DORMANT PATATES YUMRULARINDAN, YAPRAKTAN VE AFITTEN (*MYZUS PERCICAE* SULZER) PATATES YAPRAK KIVIRCIKLIK VIRÜSÜNÜN RT-PCR ILE BELIRLENMESI

Marfona, Agria ve Granola çeşitlerine ait dormant yumrulardan, yaprak örneklerinden, sera şartlarında yetiştirilen PLRV ile enfekteli bitkilerde beslenen şeftali yaprak biti (*Myzus percicae* Sulzer) ile tarladaki sarı su tuzaklarından toplanan kanatlı afitlerden PLRV'nin belirlenmesinde RT-PCR tekniği kullanılmıştır. Bu amaç için, her bir çeşitten 60 yumru olmak üzere toplam 180 yumru testlendikten sonra, pozitif ve negatif sonuç veren bütün yumrular sonuçların mukayesesi için serada saksılara dikilmiş ve tekrar yapraklardan RT-PCR yapılmıştır. Marfona, Agria ve Granola çeşitlerinden rasgele seçilen 60'şar yumrudan sırasıyla 14, 18 ve 22 yumrunun PLRV ile enfekteli olduğu belirlenirken; aynı yumrular dormansileri kırıldıktan sonra serada yetiştirilip, bu bitkilerden alınan yaprak örnekleri testlendiğinde PLRV'nin enfeksiyon oranı Marfona, Agria ve Granola için sırasıyla 16, 22 ve 28 olarak belirlenmiştir. Diğer taraftan, PLRV ile enfekteli olduğu belirlenen seradaki bitkilerde beslenen ve sonra tek ya da grup olarak toplanan şeftali yaprak biti (*Myzus persicae* Sulzer) RT-PCR yapıldığında hem tek hem de gruptan (50 afitten) PLRV belirlenebilirken; patates tarlalarından toplanan afitlerden nested RT-PCR uygulamasına rağmen bu virüs belirlenememiştir.

Anahtar Kelimeler: PLRV, dormant patates yumrusu, yaprak, afid, RT-PCR

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Detection of Viruses in Potato Leaves, Seed Tubers and Weeds by ELISA in Tokat Province, Turkey

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ABSTRACT

To detect potato virus diseases and find out their distribution in Tokat province, 168 plant samples were collected from the fields in twelve villages of Central, Niksar, Erbaa and Artova districts in 2001. According to the survey results, *Potato virus X* (PVX) and *Potato virus S* (PVS) were detected in 22.62% and 20.83% by DAS-ELISA (Double antibody sandwich- enzyme linked immunosorbent assay), respectively. Inspite of *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) were not detected in single infections, they were found as mixed infections. The infection rates of PVX+PVS, PVS+PVX+PLRV, PVY+PLRV and PVS+PVX+PVY were 26.19%, 5.95%, 2.98% and 2.98%, respectively.

Another aim of this study was determining distribution rates of potato viruses in seed potato tubers used for planting material in Niksar, Erbaa, Artova, Pazar and Başçiftlik districts of Tokat under the greenhouse conditions in 2002. When 159 leaf samples were tested by using DAS-ELISA, the results showed that infection rates of PVS, PVX, PVY and PLRV were 15.76%, 3.03%, 10.91% and 2.42%, respectively. The most prevalent viruses in mixed infections were PVS+PVY with 23.64% infection ratio. Other mixed infections were PVS+PVX (7.27%), PVS+PVX+PVY (4.85%), PVS+PVY+ PLRV (3.64%), PVS+PVX+PVY+PLRV (3.03%), PVX+PVY (2.42%) and PVY+PLRV (0.61%) in potato tubers.

Cuscuta sp. and *Abutilon theophrastii* Medik. were found to be alternative host of PVS and PVY in the region, respectively.

Key words: Potato, PVS, PVX, PVY, PLRV, weed

DETECTION OF VIRUSES IN POTATO LEAVES, SEED TUBERS AND WEEDS BY ELISA IN TOKAT PROVINCE, TURKEY

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important industrial crop and human food in the world (Esendal, 1990). Turkey needs approximately 125.000- 150.000 tons potato seeds per year and there is no state company that produce disease-free seed potato in Turkey. Therefore, seed potatoes have been imported, multiplied and then have been distributed to the producers by private companies (Bostan and Haliloğlu, 2004). Additionally, seed potatoes are tubers harvested and saved by farmers in the preceding season. These farm-saved potatoes generally are used.

Commercial production of potato is primarily through vegatative propagation by means of lateral buds formed on the tuber. For this reason, through such vegatative propagation many diseases are transmitted from generation to generation and region to region (Hooker, 1981). One of the most important factors which limits the productivity of potato is diseases. Virus diseases are major cause of economic losses in potato production around the world, more than 30 viruses known to infect potatoes (Salazar, 1996) and thirteen of these viruses are transmitted by aphids (Brunt, 2001). Under field conditions, potato often becomes infected with several viruses during a growing season (McDonald, 1984), resulting in decreased yield and quality of planting material (seed tubers). The most common viruses affecting potato crops singly or in combination, include *Potato virus S* (PVS, a *Carlavirus*), *Potato leafroll virus* (PLRV, a *Polerovirus*), *Potato virus X* (PVX, a *Potexvirus*) and *Potato virus A* and *Y* (PVA, PVY, both *Potyviruses*) (Singh, 1999).

There has been some studies on potato virus diseases, dealing with distribution, incidence and identification in Turkey (Çıtır, 1982; Yılmaz et al., 1990; Gümüş and Erkan, 1998; Yardımcı and Bostan, 1999; Çıtır et al., 1999; Bostan and Açıkgöz, 2000; Demir et al., 2001; Bostan and Haliloğlu, 2004; Arlı Sökmen et al., 2005). Until now, one study of viruses infecting potato has been done in Tokat province. That study used symptomatical observation, biological and serological test to show that seed potatoes were infected with pyhtoplasma, PLRV, PVY and PVX. These outhors indicated that only 10.40% of the seed potato were free of these diseases (Çıtır et al., 1999).

Despite many works carried on potato viruses in different areas of Turkey, no data about the incidence and distribution of these viruses in potato growing areas in Tokat was available. Therefore, our study was conducted to identify the major virus diseases in both field growing plants and also in seed potatoes and their alternative host weeds in Tokat province by DAS- ELISA.

MATERIALS and METHODS

Survey and sample collection: Fields were selected randomly from various locations in 12 villages in Central, Niksar, Erbaa and Artova districts of Tokat. Leaf

samples were collected from May through July in 2001. Samples were collected randomly along field diagonals, and two or three leaves from the shoot apex of each plant were taken. Symptoms of plants were recorded before putting leaf samples into plastic bags and storing in a freezer (-20°C).

Potato store surveys were selected randomly various locations in 12 villages in Niksar, Erbaa, Artova, Pazar and Başçiftlik districts of Tokat province February-March in 2002. A total of 38 stores were surveyed and 3800 tuber samples were randomly collected during the surveys. These tubers were used to make subsamples due to a limited supply reagents and greenhouse place. The number of samples taken from each store was reduced about 1/20. Subsamples consisted of 190 tubers, all of which were planted into 30-cm-diameter plastic pots containing soil mix and maintained in a greenhouse. These plants were harvested 2 months after planting. Symptoms of plants were recorded before putting leaf samples into plastic bags and storing in a freezer (-20°C) until ELISA tests were done.

Weed Material: 29 different weed species collected from potato growing areas near Central district of Tokat povince during March and August 2001. The weed species were identified at University of Gaziosmanpasa, Faculty of Agriculture, Department of Plant Protection. Each weed sample was put on polyethylene bag after being labelled. These samples were kept at deep freeze at -20°C until ELISA tests were done.

Serological Tests: DAS-ELISA method was performed by standard procedure (Clark and Adams, 1977) and instructions of the antiserum producer (Loewe Biochemica, Sauerlach, Germany) with minor changes (Arif et al., 1994) using PVS, PVX, PVY and PLRV polyclonal antiserum. Absorbance values were read at 405 nm using a microplate reader (Tecan Spectra II, Grödig/Salzburg, Austria). Samples were considered to be positive when the absorbance at 405 nm (A405) values exceeded the mean of the negative controls by at least a factor of two (Arlı Sökmen et al., 1998).

RESULTS and DISCUSSION

Virus detection and occurrence in potatoes: A total of 168 plant samples were randomly collected from the fields in Centre, Niksar, Erbaa and Artova districts of Tokat province during the surveys. In general, we observed that mild to severe mosaic, veinal necrosis, curling, chlorosis, reduced leaf size and stunded of plants in these surveys. These symptoms on potato plants observed under field conditions were similar to previous reports for potato viruses (Hooker, 1981; Anonymous, 2000; Taliansky et al., 2003). All plants were tested by DAS-ELISA for PVX, PVY, PVS and PLRV. The result of serological tests showed that potato plants were infected with four these viruses in Tokat province. The absorbance values of negative controls varied from 0.065 to 0.158 at 405 nm, depending on used antiserum for PVX, PVY, PVS and PLRV. Positive samples gave absorbance values of 0.152 to 3.748 (Table 1).

Table 1. Range of absorbance values of negative and positive controls, and positive and negative samples.

	Absorbans Values			
Virus[a]	Negative Controls[b]	Positive Controls[b]	Positive samples[b]	Negative Samples
PVS	0.073-0.158 (8)	2.242-3.964 (8)	0.192-3.748	0.091- 0.190
PVX	0.068-0.094 (8)	0.833-2.893 (8)	0.152-2.739	0.089- 0.150
PVY	0.076-0.096 (8)	0.794-1.766 (8)	0.169-1.502	0.087-0.165
PLRV	0.065-0.128 (8)	0.456-0.889 (8)	0.166-0.452	0.090-0.160

[a] *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV).
[b] Numbers in paranthesis indicate the numbers of positive and negative controls determined in the tests.

Table 2. Occurrence of viruses in samples collected during surveys from 12 villages in Tokat province.

Districts, villages	No. of samples tested	PVS	PVX	PVY	PLRV	PVS+ PVX	PVY+ PLRV	PVS+ PVX+ PVY	PVS+ PVX+ PLRV
Niksar									
Gurcesme	20	4	0	0	0	4	0	0	10
Mahmudiye	24	12	0	0	0	12	0	0	0
Haydarbey	19	0	0	0	0	7	0	0	0
Kavsak	3	3	0	0	0	0	0	0	0
Erbaa									
Cevresu	19	0	10	0	0	0	0	0	0
Kizilcubuk	14	0	14	0	0	0	0	0	0
Yukarı Candır	5	0	5	0	0	0	0	0	0
Artova									
Madas	9	0	0	0	0	6	0	0	0
Asagi Guclu	13	8	0	0	0	5	0	0	0
Yagci Musa	4	0	0	0	0	4	0	0	0
Celikli	13	0	3	0	0	0	5	5	0
Centre									
Camlibel	25	8	6	0	0	6	0	0	0
Total	168	35	38	0	0	44	5	5	10
Percent Infected	81.55	20.83	22.62	0	0	26.19	2.98	2.98	5.95

The incidence of these viruses in commercially grown potato plants is shown in Table 2. At the end of this study, PVX and PVS were detected in 22.61% and 20.83% by DAS-ELISA, respectively. In the research area, although PVY and PLRV were not detected in single infections, both viruses were detected in mixed infections with the infection rate of 2.98%. Out of the 165 plants, 47 had double virus infections. The most common double infection was PVX+PVS (26.19%). The results of ELISA showed that 5 plants (2.98% of the samples) were infected with PVS+PVX+PVY and 10 plants

(5.95% of the samples) were infected with PVS+ PVX+PLRV (Table 2). Çıtır et al. (1999) also found that PVX was prevalent virus in Tokat province. In the survey conducted in Van province for potato viruses, PVX and PVY were found in single infections. But, PLRV and PVA were detected as mixed infection with PVX+PVY (Demir et al., 2001). Yardımcı and Bostan (1999) found that PLRV was the most prevalent virus (42.2%) among three viruses (PVY, PVX and PLRV) in potato growing areas in Erzurum province followed by PVX (38.3%) and PVY (7%). They observed double virus infections with PVX+PLRV (6.29%), PVX+PVY (2.96%) and PLRV+PVY (1.48%). Yılmaz et al. (1990) tested 235 and 163 samples collected from the early growing potato cultivars in Çukurova region during surveys in 1987 and 1988, respectively. PLRV was detected in 1.6-12.14% in 1987 and 0.8-17.68% in 1988.

In this study, the result of ELISA showed that the most widespread virus was PVX in Tokat province. This virus is transmitted by contact of plant parts in the field due to wind, animals or machinery, by root contact, by the cutting knife before planting and by biting insects (Bercks, 1970; Hooker, 1981). The high incidence of PVX in the region, might be due to lack of cultural precations and use of noncertified tuber for planting material by farmers. Another important virus was PVS that is transmitted by mechanically, by contact with diseased plants in nature and by aphids (Hooker, 1981). PVY and PLRV were detected in only mixed infections. Also, this result showed that PVY and PLRV incidences were lower than other potato viruses in the region. Like PVS, both viruses are transmitted by aphids; the PVY is non-circulative, while PLRV is circulative (Basky, 2002). More than 50 species of aphids have proven capable of transmitting PVY (Harrington et al., 1986). Movement of virus into a potato field from an outside inoculum source is almost exclusively by winged aphids (Boiteau, 1997). Within-field spread of PLRV is often by apterae walking of aphids from plant to plant (Hanefi et al., 1989; Hodgson, 1991). In the previous study in Tokat province, aphid species as *Macrosiphum euphorbia* Thomas, *Aphis nasturtii* Kaltenbach, *Myzus persicae* Sulz., *Acyrthosiphan pisum* (Harris), *Aphis fabae* Scopoli and *Hyadaphis* sp. were determined by employing yellow-pan trap as potota viruses vectors (Çıtır et al., 1999). This study have shown that climatic conditions might be favaurable to reproduction of the aphid vectors in the region. On the other hand, PVY and PLRV infect many important food crops (Hooker, 1981). Also, a lot of weed species have been identified as favourable hosts for PVY (Anonymous, 2000). These results are shown that control of vectors, alternative host plants and the use of certificated seed potato tubers are minimizing to virus diseases incidence in the fields.

The percentages of plants infected with at least one of four viruses (PVX, PVY, PVS and PLRV) in Niksar, Erbaa, Artova and Central districts were 78.79, 76.32, 90.90 and 76%, respectively. The results revealed that potatoes grown in commercial fields were infected with viruses high percentage in Tokat. In this surveys, PVY was only

determined in Artova distict while PLRV was detected in Niksar and Artova districts in mixed infections. Seed potato production is concentrated at high altitudes where few aphids occur (Radcliffe and Ragsdale, 2002). Although Artova is located high altitutes of Tokat province, PVY and PLRV were detected in Artova province. The reason of this might be the availability of vectors and alternative hosts for these viruses in this high altitute area. PVX was detected all over of Tokat potato production areas while PVS was found in three districts except Erbaa.

Store survey results: The investigation of viruses in seed potatoes was conducted in Niksar, Erbaa, Artova, Pazar and Başçiftlik districts of Tokat province. Randomly selected 190 tubers, all of which were planted into pots and these plants were harvested 2 months after planting. Some of the planting seed potatoes have dormant tubers. Therefore, all sowing seed potatoes did not germinate, and some plants died during growing period. Similarly, Bostan and Haliloğlu (2004) emphasized when plants infected with PVY and PLRV, some of the plants might die because of virus infection. For this reason, a total 159 leaf samples from resting plants were tested for PVY, PVX, PVY and PLRV using DAS-ELISA tests.

The ELISA results showed that seed potato tubers obtained from Tokat province were singly infected with PVS (15.76%), PVX (3.03%), PVY (10.91) and PLRV (2.42%). On the other hand, these viruses were also detected in mixed infections as PVS+PVY (23.64%) PVS+PVX (7.27%), PVX+PVY (2.42%), PVY+PLRV (0.61%), PVS+PVX+PVY (4.85%), PVS+PVY+PLRV (3.64%) and PVS+PVX+PVY+PLRV (3.03%) (Table 3). In this study, any virus symptoms were detected on seed potato tubers even if these samples gave positive results by ELISA test. Although seed potato tubers collected from Bolu province were seen any virus symptoms; PVX, PVY, PLRV and PVS were detected by ELISA (Çalı and Yalçın, 1991). These results showed that viruses in potato tubers were caused latent infections. Similarly, Arlı Sökmen et al. (2005) emphasized that PVX, PVY and PLRV have caused latent infection in potato tubers. Distribution rates of the PLRV, PVX and PVY in infected seed potato tubers were 14%, 11% and 18%, respectively in Ayvalık and Altınova potato growing areas (Gümüş and Erkan, 1998). Similarly, Bostan and Haliloğlu (2004) reported that infection rates of the seed potato tubers with PLRV and PVX were higher than the other potato viruses in Bolu, Erzurum, Nevşehir, İzmir and Niğde. Çıtır et al. (1999) found that PLRV (11.80%) was the most prevalent virus among three viruses (PVY, PVX and PLRV) in potato-growing regions in the valley and high plateau of Tokat (Kazova, Niksar, Çamiçi and Çamlıbel) for the seed potato production. On the contrary, our results showed that the distribution frequency of PVS and PVY were higher than PLRV and PVX. This study indicated that Morfona was the leading cultivar with 50% of seed potatoes (Table 3). Besides Morfona, other cultivars such as Granola, white and yellow potatoes (local cultivars) seems to be susceptible to the potato viruses in Tokat province.

Table 3. The distribution rates of PVS, PVX, PVY and PLRV on seed tubers in Tokat.

Districts, villages	Samples No.	Origin of seed potatoes	Varieties samples tested	No. of	PVS	PVX	PVY	PLRV	PVS+ PVX	PVS+ PVY	PVX+ PVY	PVY+ PLRV	PVS+PVX+ PVY	PVS+PVY+ PLRV	PVS+PVX+ PVY+ PLRV
Niksar															
Mahmudiye	1	LFS*	Morfona	5	0	0	1	0	0	0	0	0	0	0	0
	2	LFS	Morfona	5	2	0	0	0	2	0	0	0	0	0	0
	3	LFS	Morfona	5	0	0	0	0	1	0	0	0	2	0	0
Haydarbey	4	LFS	Morfona	5	0	1	3	0	0	0	0	0	0	0	0
	5	LFS	Morfona	5	1	0	0	0	0	3	0	0	0	0	0
	6	LFS	Morfona	5	1	0	0	0	0	1	0	0	0	0	0
Sahinli	7	LFS	Morfona	3	0	1	0	0	0	2	0	0	0	0	0
	8	LFS	Morfona	5	0	0	2	0	0	0	1	0	0	0	0
	9	LFS	Morfona	5	0	0	0	0	2	2	0	0	1	0	0
Erbaa															
Center	10	Bolu	Morfona	3	0	0	0	0	0	0	0	0	0	0	0
	11	Nevşehir	Granola	4	1	0	2	0	0	0	0	1	0	0	0
Kızılcubuk	12	Nevşehir	Morfona	3	0	0	1	0	0	0	0	0	0	0	0
	13	Nevşehir	Morfona	3	0	0	1	2	0	0	0	0	0	0	0
Artova															
Aşagi Guclu	14	LFS	Morfona	3	1	1	1	1	0	0	0	0	0	0	0
	15	LFS	Granola	4	1	0	1	0	3	0	0	0	0	0	0
	16	LFS	Morfona	3	0	0	0	0	0	3	0	0	0	0	0
Yagci Musa	17	Ordu	Granola	4	0	0	0	0	0	0	0	0	0	0	0
	18	LFS	Granola	4	0	0	0	0	0	0	0	0	0	0	0
Taspinar	19	Ordu	Granola	5	2	0	0	0	0	2	0	0	1	0	0
	20	Ordu	Granola	2	0	0	1	0	0	0	0	0	0	0	0
	21	Ordu	Granola	3	0	0	0	0	0	0	0	0	0	0	0
	22	Ordu	Granola	5	0	0	2	0	0	2	1	0	0	0	0
	23	Ordu	Granola	4	0	0	1	0	0	2	0	0	0	0	0
Pazar															
Tatar	24	Niksar	Morfona	5	2	0	1	0	0	0	0	0	0	0	0
	25	LFS	Morfona	4	0	1	1	1	0	0	0	0	0	0	0
Besevler	26	Niksar	Yellow pota.	5	0	0	0	0	3	1	0	0	0	0	0
	27	Niksar	White pota.	5	3	0	0	0	0	1	0	0	1	0	0
	28	LFS	Morfona	4	1	0	0	0	0	2	0	0	0	2	0
	29	LFS	Morfona	5	2	0	0	0	1	0	0	0	1	1	0
Basciftlik															
Center	30	LFS	White pota.	4	1	0	0	0	0	3	0	0	0	0	0
	31	LFS	White pota.	5	3	0	0	0	0	1	0	0	0	0	1
	32	LFS	White pota.	3	0	0	0	0	0	3	0	0	0	1	0
	33	LFS	White pota.	5	0	0	0	0	0	5	0	0	0	0	0
	34	LFS	White pota.	5	0	1	0	0	0	2	0	0	0	1	0
	35	LFS	White pota.	5	1	0	0	0	0	2	0	0	0	0	2
	36	LFS	White pota.	5	0	0	0	0	0	2	0	0	0	1	2
	37	LFS	White pota.	3	3	0	0	0	0	0	0	0	0	0	0
Tokat-Center	38	Nevşehir	Morfona	3	2	0	0	0	0	0	0	0	0	0	0
Total	38			159	28	6	16	2	12	40	3	1	7	6	5
Percent Infected		...			15.76	3.03	10.91	2.42	7.27	23.64	2.42	0.61	4.85	3.64	3.03

* LFS: Local Farm Saved

This could be related to entrance of seed potato tubers to Tokat from various sources since last six years. Previously, potato growers have selected the seed potato tubers from the preceding year crop in Tokat. This might be restricting the introduction of new viruses to the area. Also, the reactions of the commonly used potato cultivar might be the limitig factor for occurrence of other potato viruses other than PLRV in Tokat in the past.

In order to increase or at least to maintain in the same level in potato production, it is necessary that potato seed must be changed in every three years and virus-free seeds should be used for planting (Courtbaoui, 1984). In this region, generally seed potatoes are tubers which were harvested and saved by farmers in the preceding season. Indeed, our study indicated that generally these farm-saved potatoes are highly infected with different viruses for a long term used (63%) (Table 3). In order to reduce extensive yield losses due to viral diseases in subsequent potato crops, seed tubers should be tested for the presence of viruses and virus-free seed tubers should be used for planting. Beside this, the use of resistant cultivars largery protects potato plants against infections, moreover infected plants are poor virus source for vector aphids (Barker, 1987; Barker and Woodford, 1992). Like PVS, PVY and PLRV especially spread in the field by various species of aphids. For this reason, the use of resistant cultivars and vectors control might be important factors for minimizing the viruses inoculum level in Tokat province.

Weed Hosts: A total of 29 species of weed belonging to Aristolochiaceae (1), Boraginaceae (2), Caryophyllaceae (1), Chenopodiaceae (1), Compositae (4), Convolvulaceae (2), Cruciferae (1), Equisateceae (1), Gramineae (1), Leguminosae (2), Malvaceae (2), Papaveraceae (1), Polygonaceae (3), Primulaceae (1), Ranunculaceae (1), Rubiaceae (1), Scrophulariceae (1), Solanaceae (2), and one species of parasitic plants belonging to family Cuscutaceae were tested by DAS-ELISA (Table 4).

Cuscuta sp. and *A. theophrastii* were found to be alternative host of PVS and PVY, respectively (Table 4). But, these weeds did not show any visible virus symptoms in the fields. There was no evidence that any of these other weed species were involved in the spread of potato viruses inoculum in the field. In temperate climates, most of the known weed host of PVY or PLRV were annuals and transmission via true seed did not occur (Salazar, 1996). In Canada, no weed was considered a virus reservoir (Singh, 1987). The winter annuals *Capsella bursa-pastoris* L. and *Sisymbrium altissimum* L. were known as PLRV hosts, but appeared unimportant in spread of the virus to potatoes (Thomas et al., 1997; Woodford, 1988). In the Souss Valley of Morocco, *Datura stramonium* L. supported Myzus persicae populations before the winter potato crop emergence. This weed and volunteer potatoes were implicated as being principal source of both viruliferous aphids and PLRV inoculum (Hanafi et al., 1995). But, our result dealing with *D. stramonium* showed that this species was not involved in the spread of

Table 4. The results of ELISA for different weed species being host of the potato viruses .

Family, Species	PVS	PVX	PVY	PLRV
Aristolochiaceae				
Alistolochia maurorum L.	-	-	-	-
Boraginaceae				
Buglossoides arvensis (L.) Johnst.	-	-	-	-
Myosotis arvensis (L.) Hill.	-	-	-	-
Caryophyllaceae				
Vaccaria pyramidata Medik.	-	-	-	-
Chenopodiaceae				
Chenopodium album L.	-	-	-	-
Compositae				
Centaurea cyanus L.	-	-	-	-
Cirsium arvense (L.) Scop.	-	-	-	-
Xanthium strumarium L.	-	-	-	-
Xanthium spinosum L.	-	-	-	-
Convolvulaceae				
Convolvulus arvensis L.	-	-	-	-
Convolvulus galaticus L.	-	-	-	-
Cruciferae				
Sinapis arvensis L.	-	-	-	-
Cuscutaceae				
Cuscuta sp.	+	-	-	-
Equisetaceae				
Equisetum arvense L.	-	-	-	-
Gramineae				
Sorghum halepense (L.) Pers	-	-	-	-
Leguminosae				
Coronilla scorpoides (L.) Koch.	-	-	-	-
Lathyrus spp.	-	-	-	-
Malvaceae				
Abutilon theophrastii Medik.	-	-	+	-
Hibiscus trionum L.	-	-	-	-
Papaveraceae				
Fumaria officinalis L.	-	-	-	-
Polygonaceae				
Polygonum aviculare L.	-	-	-	-
Polygonum convolvulus L.	-	-	-	-
Polygonum hydropiper L.	-	-	-	-
Primulaceae				
Anagallis arvensis L.	-	-	-	-
Ranunculaceae				
Ranunculus arvensis L.	-	-	-	-
Rubiaceae				
Galium aperina L.	-	-	-	-
Scrophulariceae				
Veronica hederifolia L.	-	-	-	-
Solanaceae				
Datura stramonium L.	-	-	-	-
Solanum nigrum L.	-	-	-	-

PLRV as virus source in Tokat province. In these surveys, there was a lot of weed species growing in potato fields since weed control in potato fields in the region was enough done by growers. For this reason, eradication of weeds in around potato fields can limit potato viruses because it has a natural host range restricted to the potato. As a conclusion, furher studies need to be searched the presence of potato viruses in alternative host weeds that serve as vectors and viruses reservoirs.

ÖZET

TOKAT İLİNDE PATATES YAPRAKLARI, TOHUMLUK YUMRU ve YABANCI OTLARDA VİRÜSLERİN ELISA YOLU İLE BELİRLENMESİ

Tokat ilinde patates virüs hastalıkları ve bunların yayılışlarını belirlemek amacıyla, 2001 yılında Merkez, Niksar, Erbaa ve Artova ilçelerine bağlı 12 köyden 168 adet bitki örneği toplanmıştır. Bu sürveyler sonucunda, DAS-ELISA yöntemi ile %22.62'sinin *Patates X virüsü* (PVX) ve %20.83'ünün *Patates S virüsü* (PVS) ile bulaşık olduğu belirlenmiştir. Yörede Patates Y virüsü (PVY) ve *Patates yaprak kıvırcıklık virüsü* (PLRV)'ne tek olarak rastlanılmamasına karşın, karışık enfeksiyonlarda bu virüslerin varlığı tespit edilmiştir. PVX+PVS, PVS+PVX+PLRV, PVY+PLRV ve PVS+PVX+PVY'nin enfeksiyon oranları sırası ile %26.19, %5.95, %2.98 ve %2.98'dir.

Bu çalışmada ayrıca, 2002 yılında Tokat ilinin Niksar, Erbaa, Artova, Pazar ve Başçiftlik ilçelerinde ekim materyali olarak kullanılan tohumluk patateslerdeki virüslerin bulunma oranının sera şartlarında belirlenmesi amaçlanmıştır. 159 adet yaprak örneği DAS-ELISA ile testlenmesi sonucunda; PVS, PVX, PVY ve PLRV'nin enfeksiyon oranlarının sırası ile %15.76, %3.03, %10.91 ve %2.42 olduğu görülmüştür. Karışık enfeksiyonlarda en yaygın virüsler %23.64 enfeksiyon oranı ile PVS+PVY'dir. Patates yumrularında diğer karışık enfeksiyonlar ise PVS+PVX (%7.27), PVX+PVY (%2.42), PVY+PLRV (%0.61), PVS+PVX+PVY (%4.85), PVS+PVY+PLRV (%3.64) ve PVS+PVX+PVY+PLRV (%3.03)' dir.

Yörede, *Cuscuta* sp. PVS'nin ve *Abutilon theophrastii* Medik. PVY'nin alternatif konukçuları olarak bulunmuştur.

Anahtar Kelimeler: Patates, PVS, PVX, PVY, PLRV, yabancı ot

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Factors Affecting Detection of ACLSV and ApLV from *Prunus Genus* by Reverse Transcription Polymerase Chain Reaction

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ABSTRACT

Reverse transcription polymerase chain reaction (RT-PCR) is becoming a convenient screening assay in research laboratories at RNA level. RT-PCR based diagnostic testing requires specificity, sensitivity and reproducibility. We have examined the affect of critical RT-PCR parameters on reliable detection of plant viruses. A peach isolate of *Apple chlorotic leaf spot virus* (ACLSV) and an apricot isolate of *Apricot latent virus* (ApLV) were used as reference isolates in RT-PCR reactions. According to results obtained, inadequate amount of Taq DNA polymerase enzyme yielded no amplification product. Likewise, inadequate dNTP concentration resulted detrimental effect on PCR yield. Determination of the optimum primer concentration was proved to be of prime importance in a given RT-PCR reaction. The reduction of reverse transcriptase enzyme concentration yielded high quality of PCR products. In RT-PCR reaction performed in the absence of RNase inhibitor resulted reliable detection bands.

Key words: RT-PCR, PCR parameters, optimization

INTRODUCTION

The discovery of the repeated rounds of DNA synthesis with a thermostable DNA polymerase enzyme has helped the scientists to use powerful technique known as polymerase chain reaction (PCR). The technique has become a standard laboratory technique and has been successfully utilized in plant virology mainly in virus detection studies (Taylor, 1991). In detection of plant RNA viruses by PCR an additional step is needed known as complementary DNA synthesis. The combination of reverse transcription and PCR technologies is referred to as RT-PCR. The technique can be used to determine the presence of or absence of a transcript, to estimate expression levels and to clone cDNA products. Of PCR methods, RT-PCR is the most sensitive and versatile. Since RNA cannot serve as a template for PCR, reverse transcription is combined with PCR to make complementary DNA (cDNA) (Anonymous, 1995).

RT-PCR has the potential to be an extremely sensitive alternative to ELISA, providing a means for detecting ACLSV in woody plants throughout the year (Kawasaki, 1990; Rowhani et al., 1993) even during seasons of low titer (Rowhani et al., 1998). The technique has some encountered problems which include: no detectable product or a low yield of the desired product, the presence of nonspecific background bands due to mispriming or misextention of the primers, and the formation of primer-dimers that compete for amplification with the desired product (Innis et al., 1990).

Alteration of PCR components such as PCR buffer constituents, dNTPs, and enzyme concentrations in uniplex PCRs usually results in little, if any, improvement in the sensitivity or specificity of the test. Increasing the concentration of these factors may increase the likelihood of mis-priming with subsequent production of spurious nonspecific amplification products (Elnifro et al., 2000). Increasing the concentration of PCR parameters may increase the likelihood of mispriming, with subsequent production of nonspecific amplification products. However optimization of those components in multiplex PCRs that are designed for simultaneous amplification of multiple targets is usually beneficial. Variation in concentrations of reaction components greater than those used in uniplex PCR reflects the competitive nature of the PCR process (Markoulatos et al., 2002).

Apricot Latent virus (ApLV) was fist described in Moldova in apricot, which was introduced from Bulgaria (Zemchik and Verderevskaya, 1993). The virus infects the apricots with no apparent symptoms and detectable by molecular tools mainly by RT-PCR (Nemchinov and Hadidi, 1998). Apple Chlorotic Leaf Spot Virus (ACLSV) was first isolated from apple trees by Mink and Shay (1959). ACLSV appears to be world-wide in occurrence in deciduous tree fruits and ornamentals (Chairez and Lister, 1973). Due to its cosmopolitan distribution ACLSV is considered one of the most economically important viruses affecting both stone and pome fruits (Martelli et al, 1994).

Relatively little has been published about the critical factors and common difficulties frequently encountered with RT-PCR in detecting plant viruses. Successful diagnostic testing needs specificity, sensitivity and reproducibility. By changing proportions of components PCR characteristics can be optimized. In this article we briefly discuss the effects of the main PCR components in RT-PCR reaction to allow reproducible results in detecting ApLV and ACLSV.

MATERIALS and METHODS

Virus source. A peach (*Prunus persica* L.) isolate of ACLSV collected from Van and an apricot (*Prunus armeniaca* L.) isolate of ApLV (Gümüş et al., 2004) supplied from İzmir were used as reference isolates in the course of trials. Leaf tissues of one-year old shoots of systemically infected peach and apricot were used in RT-PCR assays.

Plant total RNA extraction and synthesis of complementary DNA (cDNA). Total RNA extraction of both viruses was performed according to method described by Foissac et al., (2000). To study RT-PCR characteristics oligonucleotide primer sequences 5′-TTCATGGAAAGACAGGGGCAA -3, 5′-AAGTCTACAGGCTATTTATTATAAGTCTAA-3 reported by Menzel et al., (2002) were used to perform RT-PCR. The amplified fragment was in length of 677 bp. The primer sequence of ApLV was 5'-GGAATAGAGCCCCAAGAAG-3' and 5'-AGCAAGGTAAACGCCAAC-3' which led to the amplification of the expected size specific product (200bp) from ApLV infected tissue (Nemchinov and Hadidi, 1998). The sequences of reverse complementary primers were used for both viruses in cDNA synthesis.

cDNA Synthesis. Purified RNA was used as template to generate the single stranded cDNA. To a nuclease free microsantrifuge tube 2 µl of template RNA of ACLSV or ApLV, 1 µl (100 pmol/µl) of each specific primer and 1 µl of 10 mM dNTP (10 mM each dATP, dGTP, dCTP and dTTP) were added and completed to 12 ml with sterile distilled water. The mixture then heated to 65°C for 5 min and quickly chilled on ice. Then 4 µml of 5X first strand buffer, 2 µl of 0.1 M DDT and 1 µl of RNase inhibitor (40 units/µl) were added and mixed gently and spined briefly. Mix contents of the tube incubated at 42°C for 2 min and 1ml of Reverse transcriptase (Fermentas) was added and incubated at 42°C for 50 min. For the inactivation of reverse transcriptase (RT) the mixture was then heated to 70°C for 15 min.

PCR amplification. Two micro liter of cDNA was mixed with 48 µl of the amplification mixture containing 5 µl of the 10X reaction buffer (200 mM Tris-HCL pH:8.4, 500 mM KCl) 3 µl of $MgCl_2$ (25 mM), 1 µl dNTPs (10 mM), 1 µl of each primer (100 pmol/µl), 0.4 µl of *Taq* DNA polymerase (Fermentas) and 36.6 µl of RNase free sterile water. Initial denaturation was at 94°C for 3 min and followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 5 min for ACLSV (Menzel et al., 2002). To amlify ApLV by PCR the cycling parameters were as follows: 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension 72°C for 45 sec and final extension 72°C for 7 min (Nemchinov and Hadidi, 1998). 10ml of amplified PCR product was analyzed by agarose gel electrophoresis and ethidium bromide staining. While one of the PCR parameters was increased stepwise the others were kept constant in the optimization of PCR parameters.

RESULTS and DISCUSSION

A peach isolate of ACLSV and an apricot isolate of ApLV were served as reference isolates to assess the impact of the PCR components (primer, cDNA, dNTP, and *Taq* DNA polymerase, reverse transcriptase and RNase inhibitor) to the RT-PCR reaction. According to our experience individual reaction component concentrations should be adjusted for efficient amplification of specific targets. When starting a PCR

reaction priority should be given to adjusting primer concentration. As shown in the Figure 1a, the gradually decrease in standard primer concentration which commonly supplied by commercial sources have positively affected the quality of the PCR products.

In the course of trials it was frequently encountered that the use of recommended concentration of a given commercial primer always resulted poor yield in a RT-PCR reaction. Reduced concentrations were found to be safer in the obtaining better results. As seen in Fig. 1a standard primer concentration (lane 1) increased the probability of spurious priming and led the reduction of amplification yield. Innis et al., (1990) suggested that regardless the primer choice, the final concentration of the primer in any reaction must be optimized.

The cDNA template concentration was one of the most crucial components in the RT-PCR system. Photometric measurement of cDNA gave 165 ng/µl which corresponds 330 ng for 2 µl of target DNA (Fig 1b, lane 5). The cDNA quantities well above 660 ng/µl (4 µl cDNA) hampered PCR reaction rapidly. Following the primer optimization the arrangement of cDNA seems to be necessary in PCR optimization studies.

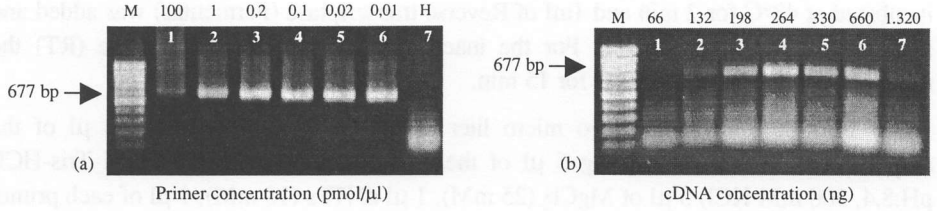

Figure 1. The affect of primer (a) and cDNA (b) concentration on RT-PCR reaction, M: molecular size markers, H: healthy control.

The four dNTPs were used at equivalent concentrations to minimize misincorporation errors. As shown in Fig. 2a 1-10 mM deoxynucleotide concentrations was sufficient to amplify the reverse transcribed genome of ACLSV. The concentrations lower than 1 mM resulted no amplification yield. Innis et al., (1988) proposed that low dNTP concentrations minimize mispriming at nontarget sites and reduce the likelihood of extending misincorparated nucleotides. Henegariu et al., (1997) reported that the best results were obtained 200 and 400 µM each dNTP, values above which the amplification was rapidly inhibited.

When dNTP concentration was reduced stepwise from 10-0,0002 mM the amplification was rapidly inhibited. dNTP concentration lower than 5 mM allowed PCR amplification but with visibly lower amounts of PCR products. Cobb and Clarkson, (1994) was reported that the higher concentrations of dNTPs promoted amplification of spurious products.

In another test aimed at examining the proper *Taq* DNA concentration, an RT-PCR using ACLSV primer mixture was performed. The concentration of other parameters was kept constant while the enzyme concentration was reduced stepwise from 5 to 0.001 unite/µl (Fig. 2b). The best results were achieved at 5, 4, 3, 2 and 1 unite/µl enzyme concentrations. No significant difference was found between 5 and 1 unite/µl enzyme concentrations. The enzyme concentrations, between 0.5 and 0.16 unite/µl, still allowed PCR amplification, but with somewhat less efficiency. In enzyme concentrations lower than 0.16 unite/µl, the reaction was rapidly inhibited (Fig. 2b, lane 9).

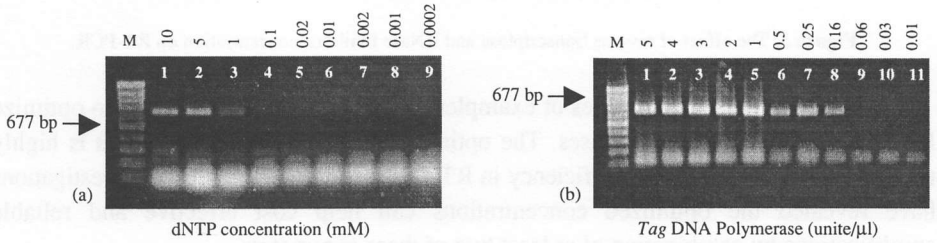

Figure 2. The affect of dNTP and polymerase anzyem concentration on RT-PCR reaction.

One important observation, coming from experiments with RT-PCR, is that the enzymes (reverse transcriptase and *Taq* DNA polymerase) used in the tests were determined as the main factors resulting non-specific background products. As seen in Fig. 2b in concentrations of *Taq* DNA polymerase enzyme lower than 1 unite/µl relatively clear background was obtained. Higher enzyme concentrations were resulted the accumulation of non-specific background products (conspicuous at lanes between 1-5 in Fig. 2b). Too low concentrations between 0.001-0.006 unite/µl were resulted in insufficient amount of desired products (lane 9, 10, and 11). Innis et al., (1990) stated that if the enzyme concentration is too high, nonspecific background products may accumulate, and if too low, an insufficient amount of desired products is made.

The effect of reverse transcriptase (RT) and RNase inhibitor in a RT-PCR system was investigated in detection of ApLV in combination of each parameter. As shown in Fig. 3 (lane 3 and 6) diluted RT was resulted better amplification yield. The lack of RNase inhibitor was not affected the PCR results even in the presence of diluted RT enzyme. According to the results obtained, it is strongly recommended to reduce RT enzyme to lower the cost of the tests and to have clear bands as well.

No significant difference was observed between diluted and undiluted RT enzyme reactions. As seen in Fig. 3 (lane 3 and 6) when RT enzyme was diluted relatively clear bands were obtained. This suggested that the amount of RT enzyme has been affected the clearance of the lanes. Even standard concentrations of RT has been causing dirty backgrounds (Fig 3. lanes 1, 2, 4 and 5).

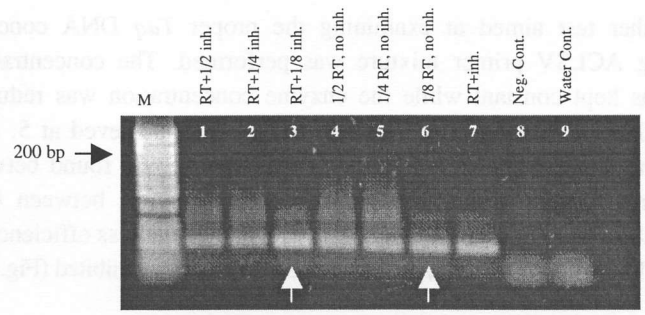

Figure 3. The affect of reverse transcriptase and RNase inhibitor concentration on RT-PCR.

We have presented a series of examples of testing various parameters to optimize RT-PCR in detecting plant viruses. The optimization of RT-PCR parameters is highly needed to have sensitivity and efficiency in RT-PCR reactions. The present investigations have revealed the optimized concentrations can help cost effective and reliable amplifications by combination of at least two of these parameters.

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ÖZET

REVERZ TRANSKRİPTAZ POLİMERAZ ZİNCİR REAKSİYONU YÖNTEMİ İLE ACLSV VE ApLV VİRÜSLERİNİN *PRUNUS* GENUSUNDAN TEŞHİSİNİ ETKİLEYEN FAKTÖRLER

Reverz transkriptaz polimeraz zincir reaksiyonu (RT-PCR) patoloji laboratuarlarında yürütülen RNA tabanlı araştırma çalışmalarında yaygınlık kazanan bir yöntem olmaya başlamıştır. RT-PCR temelli teşhis çalışmaları spesifite, hassasiyet ve yinelenebilirlik özellikleri gerektirmektedir. Bu çalışmada RT-PCR parametrelerinin bitki virüslerini teşhisindeki etkileri araştırılmıştır. RT-PCR testlerinde bir şeftali izolatı olan *Apple chlorotic leaf spot virüs* (ACLSV)'ü ile bir kayısı izolatı olan *Apricot latent virüs* (ApLV)'ü referans izolatlar olarak kullanılmıştır. Elde edilen bulgular doğrultusunda çok düşük *Taq* DNA polimeraz enzimi konsantrasyonu yeteri kadar amplifikasyon ürünü oluşmamasına neden olmuştur. Benzer şekilde yetersiz dNTP konsantrasyonu PCR reaksiyonunu olumsuz etkilemiştir. Optimum primer konsantrasyonunun belirlenmesi PCR reaksiyonundaki en önemli

adımlardan biri olarak belirlenmiştir. Reverz transkriptaz enzimi konsantrasyonun azaltılması PCR ürünlerinin kalitesini olumlu yönde etkilemiştir. RNase inhibitörü eksikliğinde gerçekleştirilen RT-PCR reaksiyonu sonucunda güvenilir teşhise yetecek bantlar elde edilmiştir.

Anahtar Kelimeler: RT-PCR, PCR parametreleri, optimizasyon

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Effects of Salicylic Acid, Harpin and Phosphorus acid in Control of Late Blight (*Phytophthora infestans* Mont. De Barry) Disease and Some Physiological Parameters of Tomato

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ABSTRACT

Plant disease resistance elicitors, salicyclic acid and $Harpin_{Ea}$ alone and in combination with systemic fungicide phosphorus acid (Agrifos 400) were applied to tomato plants, which were inoculated with *Phytophthora infestans* after treatments. The average disease reduction was about 47% after treatment with SA and 55% with $Harpin_{Ea}$. One of the newest promising fungicide Agrifos 400 controlled the late blight efficiently with 88% compare to untreated ones. The combinations of SA and HarpinEa with fungicide were found much more effective than that of single treatments. While SA induced the accumulation of pathogenesis related proteins (PR proteins), chitinase, β-1,3-glucanase and peroxidase, $Harpin_{Ea}$ was failed to provide significant increase in chitinase activity. SA and $Harpin_{Ea}$ treatments also exerted pleiotropic effects on tomato growth and development and photosynthetic pigment contents. Surprisingly, fungicide application induced the disease resistance components like the elicitors used in our experiment. The results confirmed that Agrifos 400 is not only effective fungicide against late blight but also a plant activator.

Key words: Salicylic acid, Harpin, phosphorus acid, tomato, *Phytophthora infestans*

INTRODUCTION

Inoculation of plants with pathogens or the treatment with some chemical compounds can result in the establishment of systemic acquired resistance (SAR) (Ryals et al., 1996) which is accompanied by synthesis of pathogenesis-related proteins (PR-proteins) (Linthorst, 1991).

It has been shown that some PR-proteins have β-1,3-glucanase (EC 3.2.1.39, PR-2 group) or chitinase (EC 3.2.1.14, PR-3 group) activity at least *in vitro* (Jacobsen et al., 1990). They are suggested to play a role in the defence ability of the plant e.g., they are capable of degrading fungal cell wall polysaccharides and so could inhibit fungal

growth (Roulin and Buchala, 1995). Peroxidases (EC 1.11.1.7) have been implicated in a variety of defence-related processes, including the hypersensitive response (HR), lignifications and cross-linking of phenolics and glycoproteins (Wojtaszek, 1997).

Late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is an economically important disease of tomato. Disease management strategies primarily depend on sanitary practices and well-timed fungicide applications. However, development of fungicide resistance within populations of *P. infestans* has become a problem (Fry and Goodwin, 1997) and alternative approaches that can be incorporated into integrated pest management of tomato late blight disease are needed. SAR against late blight was previously demonstrated in tomato by inoculating *P. infestans* (Enkerli et al., 1993) or tobacco necrosis virus (TNV) (Anafoka and Buchenauer, 1997) or by applying chemicals onto tomato (Cohen, 1994) prior to challenge with pathogen (Zhinong et al., 2002).

The goal of the work presented in this paper, was to assess the efficacy of SA and $Harpin_{Ea}$ on the induction of SAR against *P. infestans* infection in tomato plants and to compare their actions with phosphorous acid, which is widely used fungicide for the disease. The purpose of the current work was also to evaluate efficacy of the product on plant growth and development and the photosynthetic metabolism.

MATERIALS and METHODS

Tomato (*Lycopersicum esculentum* Mill.) seedlings were grown under greenhouse conditions (25±2°C, 8/16 photoperiod) in 2005. The seedlings (30-day-old) were pulverized with water and treated plants were applied SA (100 mg L^{-1}, AnalaR, UK), $Harpin_{Ea}$ (0.65 g L^{-1}, Messenger, AMC Turk, Antalya-Turkey, Eden Bio Science, USA) and phosphorous acid, a systemic fungicide, (4 ml L^{-1} Agrifos 400, Agrikem, İzmir-Turkey) alone, and SA and Hrp in combination with fungicide. SA, Harpin and phosphorus acid were sprayed two times totally when they were used alone. However, each chemical was used once in alternation program.

Stock cultures of *P. infestans* were taken from Ege University, Faculty of Agriculture, Department of Plant Protection. Conidia suspensions of *P. infestans* at 10^6 spore concentration were artificially inoculated in two weeks after the treatments on the leaves. The seedlings were covered with polyethylene bags for 48 h. Disease severity index (DSI) was calculated according to 0-5 scale (Anonymous, 1996) and efficacy rate was calculated by the equation of Karman (1971).

Leaf homogenates were obtained by grinding leaves in a mortar containing 0.1 M NaAc (sodium acetate) buffer (pH 5.0) (2.0 mL/g fresh weight) and were centrifuged for 15 min at 9.000 g. The protein contents were determined by dye binding method of Bradford (1976) with bovine serum albumin (Sigma, UK) used as a standard. Chitinase activities were determined by the method of Hackman and Goldberg (1964). β-1,3-Glucanase activity were determined by spectrophotometrically at 450 nm by the

method of Boller (1992). The activity of enzyme was assayed by measured the rate of reducing sugar production with laminarin (Sigma, UK) as the substrate. For peroxidase assay, the leaves were homogenized by grinding in a mortar containing 0.05 M $NaPO_4$ (sodium phosphate) buffer (pH 6.5) (2.0 mL/g fresh weight). After centrifugation, enzyme activity was assayed using pyrogallol and H_2O_2. The absorbance was spectrophotometrically measured at 300 nm (Kanner and Kinsella, 1983).

The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were measured by the spectrophotometric method (Witham et al., 1971) by using 80% acetone extraction. Harvested plants were weighted as fresh and then dried in the oven for 48 h at 105°C. Stem height, root height and total height were measured by rule. Each experimental result was analysed statistically by analysis of variance (ANOVA). Means were compared with LSD with a significance level of $P < 0.05$.

RESULTS

High disease severity with 48.4% was calculated in treated plants watered only. Plants treated with phosphorus acid, SA + phosphorus acid, and $Harpin_{Ea}$ + phosphorus acid have showed much less disease with 87.7% and 82.4% efficacies, respectively (Fig.1).

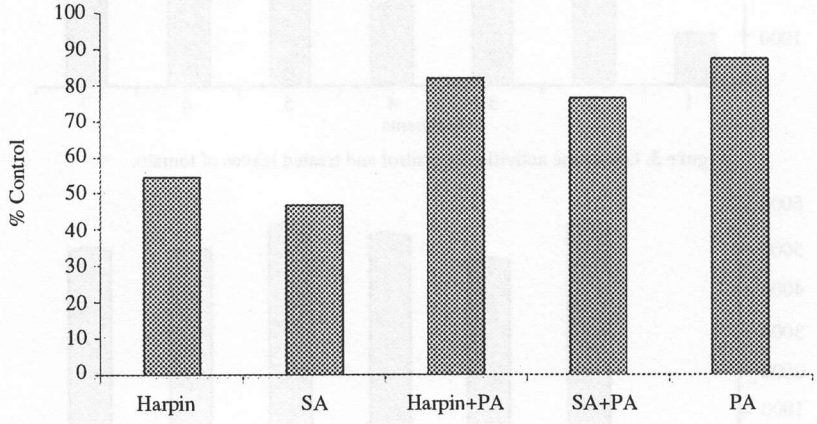

Figure 1. Effects of salicylic acid, Harpin and fungicide treatments on late blight under greenhouse conditions. SA: Salycilic acid; PA: Phosphorus acid.

Compared to distilled water treated control, SA induced the accumulation or *de novo* synthesis of chitinase (Fig.2), glucanase (Fig. 3), and peroxidase (Fig. 4), while $Harpin_{Ea}$ was failed to provide significant increase in chitinase activity. All leaf samples that were collected after chemical treatments, showed significant increased in β-1,3-glucanase activity when compared to the negative control (Fig. 3). $Harpin_{Ea}$ combination with phosphorus acid led to highest enhancement of peroxidase activity as much as 187-fold (Fig. 4).

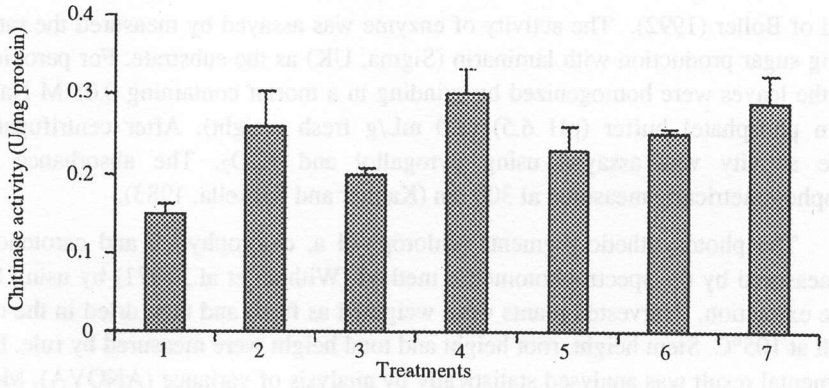

Figure 2. Chitinase activities of control and treated leaves of tomato.

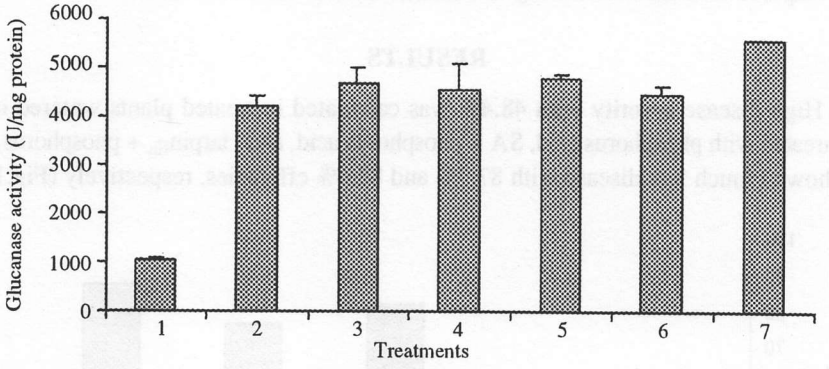

Figure 3. Glucanase activities of control and treated leaves of tomato.

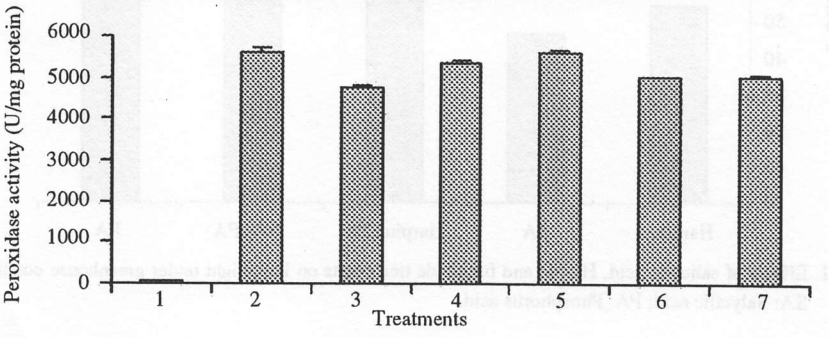

Figure 4. Peroxidase activities of control and treated leaves of tomato.

1. Control; 2. Pathogen; 3. Harpin; 4. SA; 5. Harpin + phosphorus acid; 6. SA + phosphorus acid; 7. Phosphorus acid.

Although fresh weight of root was not changed by late blight disease, significant increases were observed by SA+fungicide, $Harpin_{Ea}$+fungicide combinations and single phosphorous acid treatment with about 95.5%, 36% and 100%, respectively compared with negative control. Root dry weight was affected significantly by *P. infestans* inoculation and $Harpin_{Ea}$ treatment of tomato leaves around 50%, when compared with negative

control. Stem fresh weight showed a significant increase about 17.5% compared to negative control, however there was a significant but slight reduction at SA treated tomato plants around 5%. Other chemical treatments had no effect on stem fresh weight. Stem dry weight of tomato plant was the only affected parameter by SA treatment and a 2-fold increase was observed when compared with negative control. Regarding the root height, treatment with phosphorous acid had no influence on stem height. Total height was positively affected by SA treatment about 35.5% compared with positive control. Other chemical treatments except SA did not show any recovering effect on total height according to positive control (Table 1).

Table 1. Effect of salicylic acid, Harpin and phosphorus acid as fungicide treatment on growth and development of tomato under greenhouse conditions. Data show the averaged and ± standard errors of 10 replicates.

Treatments	Root weight (g)		Stem weight (g)		Total Height (cm)
	Fresh	Dry	Fresh	Dry	
Control	0.68 ± 0.07	0.06 ± 0.01	7.30 ± 0.29	0.59 ± 0.12	32.67 ± 3.93*
Pathogen	0.79 ± 0.09	0.09 ± 0.01*	7.22 ± 0.44	0.97 ± 0.26	22.50 ± 2.75
Harpin	0.77 ± 0.07	0.09 ± 0.06*	8.33 ± 1.00	0.79 ± 0.14	27.50 ± 0.50
SA	0.75 ± 0.09	0.07 ± 0.01	6.93 ± 0.17*	1.76 ± 0.14*	30.50 ± 3.88*
Harpin + Fungicide	0.93 ± 0.08*	0.06 ± 0.01	8.58 ± 0.49*	0.66 ± 0.13	24.33 ± 0.33
SA+ Fungicide	1.33 ± 0.06*	0.07 ± 0.03	7.66 ± 0.16	0.83 ± 0.02	28.83 ± 3.97
Fungicide	1.37 ± 0.04*	0.08 ± 0.01	8.12 ± 0.24	0.99 ± 0.11	26.00 ± 0.60

* Indicates significant differences among treatments according to a LSD test at $p<0.05$ level.

SA treatment were observed to be the most efficient treatment, causing around 50% enhancement in chlorophyll a, chlorophyll b and carotenoid contents when compared with negative control (Fig. 5). Moreover, single treatments of $Harpin_{Ea}$ and phosphorus acid, and $Harpin_{Ea}$+phosphorus acid combinations resulted in significant increase in photosynthetic pigment contents.

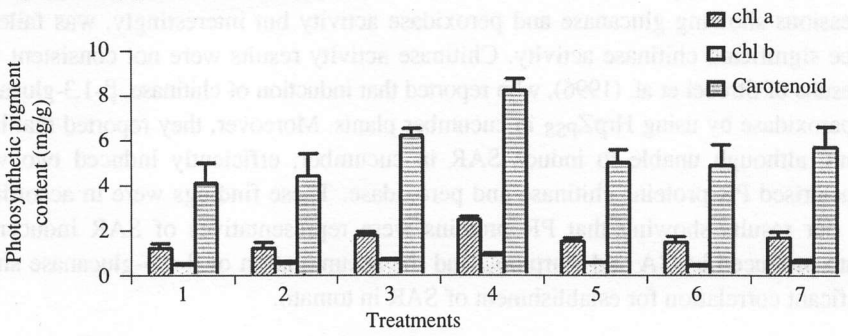

Figure 5. Photosynthetic pigment contents (mg/g) of control and treated leaves of tomato. Data show the averaged and ± standard errors of 10 replicates. 1. Control; 2. Pathogen; 3. Harpin; 4. SA; 5. Harpin+ phosphorus acid; 6. SA+phosphorus acid; 7. Phosphorus acid.

DISCUSSION

SA and $Harpin_{Ea}$ treatments elicited systemic protection against late blight under greenhouse conditions by reducing disease severity at some degrees. SA is an endogenous signalling molecule (Klessig and Malamy, 1994), which accumulates following pathogen exposure (Malamy et al., 1990; Malamy et al., 1996; Metraux et al., 1990) and is required for induction of SAR. There are no reports of exogenous SA treatment elicitation of systemic protection on tomato/*P. infestans* system. However, the induction of the resistance by DL-3-aminobutyric acid (BABA) by increasing the content of free SA in tomato leaves was revealed (Jeun et al., 2000). It is reported that higher SA concentrations had increased the resistance against late blight disease when injected into potato seed tubers before planting (Quintanilla and Brishammar, 1998). $Harpin_{Ea}$ induced disease resistance results are in agreement with previous reports of classical SAR induced by harpin in a variety of plants against a broad array of pathogens (Tosun et al., 2002; Qui et al., 1997; Wei et al., 1998). Fungicide treatment was found to be most effective for preventing late blight disease. Our results support the previous reports showing that phosphorous acid products had been extensively evaluated for control of diseases caused by *Phytophthora* spp. and other oomycetes (Heaton and Dullahide, 1990). SA and $Harpin_{Ea}$ treatment in combination with fungicide provided higher effectiveness against late blight disease than single treatments. The results of our work seem to confirm those of Agostini et al., (2003), who reported that induced resistance products were effective for disease control but they may be more useful in an integrated program with standard fungicides.

In the experiments, SA efficiently induced PR proteins including proteins displaying chitinase, β-1,3-glucanase and peroxidase activity. SA effect of inducing the PR protein, which has chitinase, β-1,3-glucanase and peroxidase enzyme activity, was well established (Ryals et al., 1996; Wobbe and Klessig, 1996; Durner et al., 1997; Bokshi et al., 2003) and our results support the previous works. $Harpin_{Ea}$ treatment elicited PR protein expressions showing glucanase and peroxidase activity but interestingly, was failed to induce significant chitinase activity. Chitinase activity results were not consistent with the results of Strobel et al. (1996), who reported that induction of chitinase, β-1,3-glucanase and peroxidase by using $HrpZ_{PSS}$ in cucumber plants. Moreover, they reported that hrpH mutant, although unable to induce SAR in cucumber, efficiently induced two well-characterised PR proteins chitinase and peroxidase. These findings were in accordance with our results showing that PR proteins were representatives of SAR inducers in tomato produced by SA and $Harpin_{Ea}$, and the accumulation of β-1,3-glucanase shows significant correlation for establishment of SAR in tomato.

Previous data suggests the involvement of reactive oxygen intermediates (Alvarez et al., 1998) and programmed cell death, calcium ion channels and protein cascades in interactions of Hrp proteins with plants (Agrios, 1997). These reports explained the

increase in peroxidase activity of $Harpin_{Ea}$ treated plants. Harpin acts through the SAR pathway by a signalling process that begins upstream of SA, leading to activation of PR genes (Dong et al., 1999). Surprisingly, fungicide application induced the disease resistance components like the elicitors used in our experiment. This induction may be related to the elicitation of phosphorous acid in systemic fungicide.

SA and $Harpin_{Ea}$ treatment compensated for the negative effects of pathogens on growth and development by their pleiotropic effects. Stem height was increased SA and $Harpin_{Ea}$ treatment, and in combination with fungicide. SA treated plants showed an increase in total height and stem dry weight. Photosynthetic pigment contents (chlorophyll a, chlorophyll b and caroteoids) were increased by SA treatment. These results indicate that SA and $Harpin_{Ea}$ have several beneficial effects including the enhancement of plant growth (Raskin, 1992; Qui et al., 1997) related to signalling pathways.

SAR elicitors SA and $Harpin_{Ea}$ applied alone were often insufficient to completely control the late blight disease caused by *P. infestans* of tomato. However, when SAR elicitors, SA and $Harpin_{Ea}$, used in alternation with suitable fungicide such as Agrifos 400, it helps disease management effectively especially minimising resistance development of pathogen to the systemic fungicides.

ÖZET

DOMATES GEÇ YANIKLIK HASTALIĞININ KONTROLÜNDE ve BAZI FİZYOLOJİK PARAMETRELERİ ÜZERİNE SALİSİLİK ASİT, HARPİN VE PHOSPHORUS ASİT'İN ETKİSİ

Bitki aktivatörleri (hastalık direnç elisitörleri), salisilik asit (SA) ve $Harpin_{Ea}$ (Messenger) tek başlarına ve fungisitle (Agrifos 400) birlikte domates bitkisi yapraklarına uygulanmış ve ardından bitkiler *Phytophthora infestans* etmeni ile inoküle edilmiştir. Ortalama etkililik SA uygulamasında %47 ve $Harpin_{Ea}$ uygulamasında ise, %55 olarak bulunmuştur. Yeni gelişen ümitvar fungisitlerden biri olan Agrifos 400 ise, kontrole göre geç yanıklık hastalığı %88 oranında control etmiştir. SA ve $Harpin_{Ea}$ ile fungisit kombinasyonları tek başlarına uygulamadan daha etkili bulunmuştur. Patojen bağıntılı (PR) proteinler, kitinaz, β-1,3-glukonaz ve peroksidaz birikimi SA tarafından uyarılırken, $Harpin_{Ea}$ kitinaz aktivitesinde önemli bir artışa neden olmamıştır. Salisilik asit ve $Harpin_{Ea}$ uygulamaları aynı zamanda domateste büyüme-gelişme ile fotosentetik pigment içeriklerinde de pleotropik etkiler göstermiştir. Fungisit uygulaması da hastalığa dayanıklılık unsurlarını denemede kullanılan elisitörler gibi uyarmıştır. Bu sonuçlar, phosphorus asit etkili maddeli Agrifos 400 isimli fungisitin sadece geç yanıklık hastalığını başarılı biçimde control etmekle kalmayıp aynı zamanda bir bitki aktivatörü olduğunu kanıtlamaktadır.

Anahtar Kelimeler: Salisilik asit, Harpin, phosphorus asit, domates, *Phytophthora infestans*, PR proteinleri

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Induction of Resistance to *Botrytis cinerea* Pers in Pepper by β-Aminobutyric Acid

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ABSTRACT

Using pepper seedlings, the nonprotein amino acid, DL-beta-amino-n-butyric acid (BABA) was assayed for its ability to induce resistance in pepper against gray mould disease caused by *Botrytis cinerea*. It was found that foliar spray of BABA to leaves controlled the gray mould infection by 59.3%. Moreover, the synergistic interaction between BABA and subsequent treatment of fungicide (fenhexamide) gave a higher protection rate reaching to 82.2% when compared with the single treatments of BABA and fungicide. The enhanced resistance of BABA-treated plants was associated with significant increases in the activities of PR proteins chitinases (PR-3), β-1,3 glucanase (PR-2) and peroxidase. Accumulation of PR proteins and peroxidase may play an essential role in BABA's possible mode of action. Besides the induction of disease resistance, BABA caused a significant increase in the content of photosynthetic pigments (chlorophyl a, chlorophyl b and carotenoids) and root heights in treated pepper plants.

Key words: Disease resistance, β-Aminobutyric acid, Pepper, *Botrytis cinerea*

INTRODUCTION

Botrytis cinerea Pers.: Fr. (telemorph: *Botryotinia fuckeliana* (de Bary) Whetzel) causes gray mould disease in a wide range of economically important plants. This necrotrophic plant pathogen often first establishes a symptomless quiescent infection, from which it later spreads rapidly after harvest to cause serious losses, particularly in fruit, vegetable and flower crops (Coley-Smith et al., 1980).

DL-Beta-amino-n-butyric acid (BABA) is a nonprotein amino acid, which induces resistance against a broad range of disease causing organisms, including fungi, bacteria, viruses and nematodes (Cohen, 2001; Cohen, 2002; Jakab et al., 2001). Most of studies describing the phenomenon of BABA-induced disease resistance were done in annual weedy plant species, especially those belonging to the *Solanaceae* family, such as tomato and potato (Cohen and Gisi, 1994, Cohen, 2002). In these families, it was

demonstrated that BABA may also induce resistance in pepper against *Pseudomaonas coscodes* (Hong et al., 1999) and *Phytophthora capsici* (Hwang et al., 1997). However, as far as we know, no report is yet avaliable regarding the effects of BABA on the induction of disease resistance in peper against *Botrytis cinerea.*

Induction of plant pathogen resistance is a complex mechanism involving the activation of various processes, including the stimulation of the hypersensitive response, build-up cell wall barriers, increased synthesis of phytoalexins and accumulation of pathogenesis-related (PR) proteins, including cell wall hydrolases (Hammerschmidt, 1999). Among PR proteins β-1,3 glucanase (PR-2) and chitinases (PR-3) possess direct antimicrobial activity by degrading cell wall components of pathogen (Van Loon, 1997). Additionally, these enzymes release oligosaccaride products from fungal cell walls, which in term may elicit secondary plant pathogen defense responses (Keen and Yoshikawa, 1983). Peroxidases have been implicated in a variety of defence related processes, including the hypersensitive response, lignification, cross linking of phenolics and glycoproteins, suberization and phytoalexin production (Nicholson and Hammerschmidt, 1992).

The goal of this study was to test the ability of BABA to induce resistance in pepper (*Capsicum annum* L.) against gray mould disease caused by *Botrytis cinerea.* We also examined the induction of pathogenesis related proteins and effected growth and photosynthetic parameters in plants treated with this material.

MATERIALS and METHODS

Pepper (*Capsicum annum*) seedlings were grown in greenhouse conditions at 25±2°C with 8/16 photoperiod. Experiments were performed with 4-week old pepper plants. Plants were sprayed with DL-beta-amino-n-butyric acid (BABA) (1000 mg L^{-1}, Sigma, USA), fungicide (fenhexamide, 1 ml L^{-1} Teldor SC 500-Bayer, Turkey) twice with one week intervals. BABA + fungicide combinations were applied by spraying BABA first and followed by the fungucide after one week. Both uninoculated and inoculated control plants were treated with distilled water. *Botrytis cinerea* were isolated from infected pepper plants and propagated potato dextrose agar. Conidial suspension was prepared in sterile distilled water by adding 0.01% Tween-80.

Two weeks after the first chemical treatments, all experimental groups except water treated uninoculated plants (as negative control) were challenged with 10^5 ml^{-1} conidia of *B. cinerea.* Plants were incubated for 7 days at 22°C and 95% relative humidity in growth chambers.

Disease severity was rated using a 0-4 scale, in which: 0=no necrosis, leaf area is completely healthy; 1=25% of the leaf area are with symptoms; 2=50% of the leaf area is with symptoms; 3=75% of the leaf area is with symptoms; 4=100% of the leaf area is

covered with *Botrytis cinerea* symptoms. A disease index was calculated as the sum of the areas of the 4 leaves. Each treatment was applied to five plants. Plants which were water-treated and inoculated by *B. cinerea* were served as positive control.

Preparation of leaf homogenates and partial purification of proteins

Leaf homogenates were obtained by grinding leaves in a mortar containing 0.1 M NaAc (sodium acetate) buffer (pH 5.0) (2.0 mL/g fresh weight). Homogenates were centrifuged for 15 min at 9.000 g. The protein contents were determined by the dye binding method of Bradford (1976) with bovine serum albumin (Sigma, UK) used as a standard.

Chitinase activities were determined by the method of Hackman and Goldberg (1964). Chitinazur (10 mg) (Sigma, UK) was used as substrate for this enzyme assay and was mixed with 1.5 ml of enzyme solution for 24 hours at 37°C. After centrifugation, the absorbance of the supernatant was determined spectrophotometrically (Unicam α, Helios) at 575 nm.

For β-1,3-Glucanase assay, after homogenisation of leaves, proteins were precipitated by acetone (80 %). Pellet was resuspended in 0.1 M NaAc buffer (pH 5.0) used for β-1,3 glucanase enzyme assay. The activity of the enzyme was determined by the method of Boller (1992) by measuring the rate of reducing sugar production with laminarin (Sigma, UK) as the substrate. The absorbance of reaction mixture was spectrophotometrically measured at 450 nm by using glucose as a standard.

For peroxidase assay, leaves were homogenized by grinding in a mortar containing 0.05 M $NaPO_4$ (sodium phosphate) buffer (pH 6.5) (2.0 mL/g fresh weight). After centrifugation, enzyme activity was assayed using pyrogallol (0.1 M) and H_2O_2 (0.1 M). The absorbance was spectrophotometrically measured at 300 nm (Kanner and Kinsella, 1983).

The photosynthetic pigment contents measurement

The photosynthetic pigments (chlorophyl a, chlorophyl b and carotenoids) of pepper leaves were measured by the spectrophotometric method of Witham et al. (1971) by using 80 % acetone extraction.

Each experimental result was analysed statistically by analysis of variance (ANOVA). Means were compared with LSD with a significance level of $p<0.05$.

RESULTS

The effect of BABA treatment on the disease severity

Pre-treatment of pepper plants with BABA, reduced the severity of the symptoms

caused by the *Botrytis cinerea*. The mean DSI did not exceed 15.4% in BABA-treated plants, and was 39.3% in control plants. The resistance was evident as a reduction in disease severity compared with control plants. It was observed that BABA treatments provided 59.3% protection rate against *B. cinerea*, BABA in combinations with fungicide gave higher protection rates reaching to 82.2%, when compared with the single treatments of BABA and fungicide (Table 1).

Table 1. Effect of BABA treatment on pepper gray mould control under greenhouse conditions.

Treatments	Disease severity index (%)	Protection rate (%)
Water (control)	0	0
B. cinerea	39.3 ± 2.8	0
BABA	15.4 ± 0.6	59.3 ± 2.9
Fungicide	12.3 ± 0.5	68.2 ± 1.3
BABA+Fungicide	6.6 ± 0.6	82.2 ± 1.6

*Standard deviation of avarega of five replications.

Induction of chitinase, β-1,3 glucanase and peroxidase activity

The basal chitinase activity of water treated (control) leaves, measured colorometrically, was 0.047 unit chitinase per mg protein (Fig. 1a). *B. cinerea* inoculated leaves showed a significant increase in chitinase activity. Although BABA and fungicide treatment to leaves did not cause a significant change in the activity, BABA + fungicide combination elicited chitinase activity about 104% relative to water treated pepper leaves.

β-1,3 Glucanase activity of water treated leaves was determined as 1766 unit per mg protein (Fig. 1b). This activity increased significantly when plants were inoculated with *B. cinerea*. All chemical treatments caused an alteration in the enzyme activity while the most effective treatment was BABA + fungicide combination which caused a level of 103% when compared to the water treated leaves.

Peroxidase activity of water treated plants were measured as 261 unit per mg protein (Fig. 1c). This activity was elicited significantly by inoculation of *B. cinerea*, BABA and fungicide treatment in about similar levels (approx. 1.5 fold). Spraying the plant with BABA + fungicide combination gave a higher enzyme activity when compared with single chemical treatments or *B. cinerea* inoculation.

Effect of BABA on plant growth and the level of photosynthetic pigments

Infection with *B. cinerea* did not affect significantly stem or root height when compared with the uninoculated control (Table 2). There were no significant differences among treatments regarding stem and total height. On the other hand, single treatment of BABA and BABA + fungicide combination led to an increase in only root height compared with *B. cinerea* inoculated plants, about 54.9% and 39.4%, respectively.

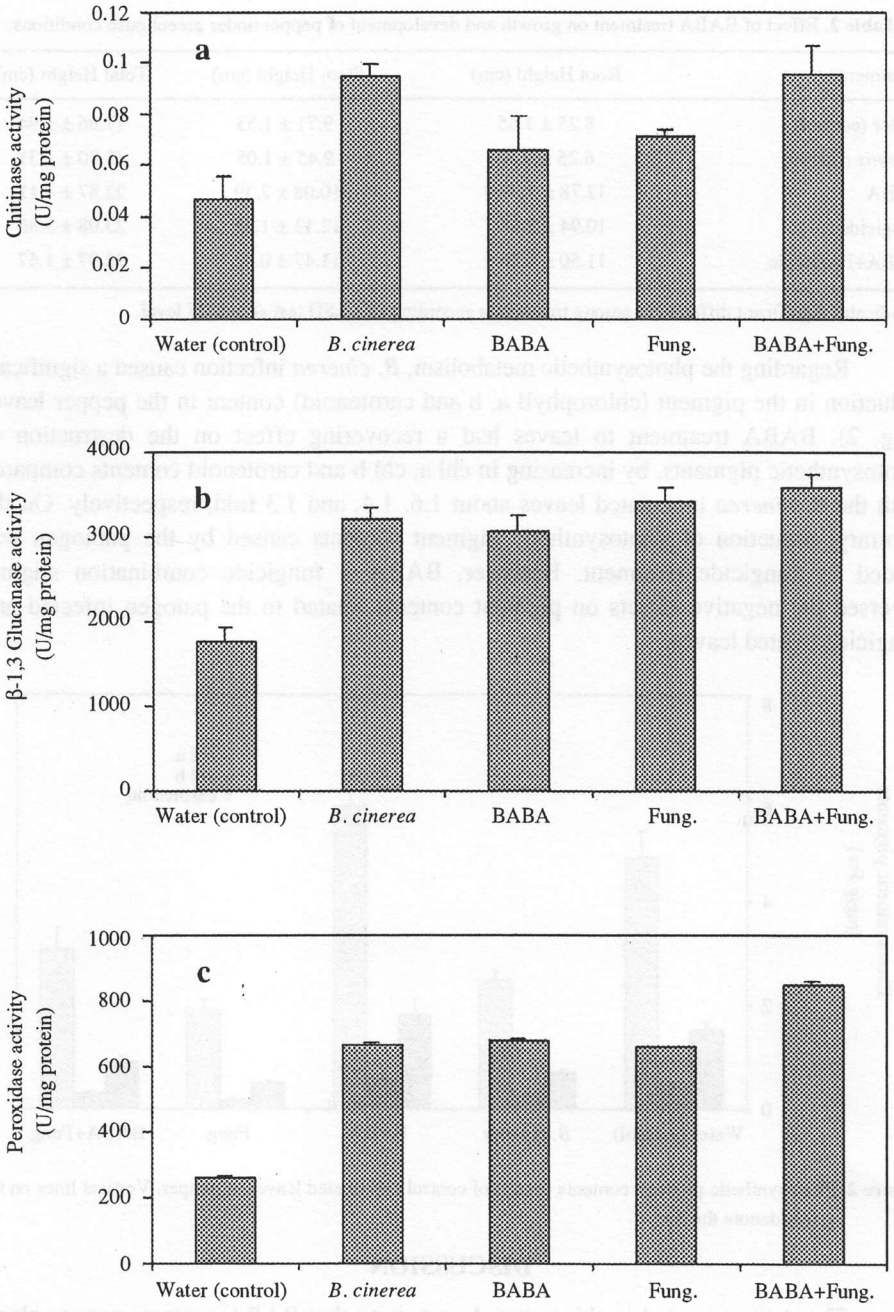

Figure 1. Detected chitinase (a), β-1,3-glucanase (b) and peroxidase (c) activities in pepper plants. Vertical lines on the graph denote the SE.

Table 2. Effect of BABA treatment on growth and development of pepper under greenhouse conditions.

Treatments	Root Height (cm)	Stem Height (cm)	Total Height (cm)
Water (control)	8.25 ± 1.65	9.71 ± 1.53	17.96 ± 2.94
Botrytis cinerea	6.25 ± 0.40	9.45 ± 1.05	15.70 ± 1.31
BABA	12.78 ± 1.99 *	10.08 ± 2.39	22.87 ± 4.11
Fungicide	10.94 ± 2.68	12.13 ± 1.35	23.08 ± 3.86
BABA+Fungicide	11.50 ± 1.28 *	11.47 ± 0.82	22.97 ± 1.67

* Indicates significant differences among treatments according to a LSD test at $p<0.05$ level.

Regarding the photosynthetic metabolism, *B. cinerea* infection caused a significant reduction in the pigment (chlorophyll a, b and carotenoid) content in the pepper leaves (Fig, 2). BABA treatment to leaves had a recovering effect on the destruction of photosynthetic pigments, by increasing in chl a, chl b and carotenoid contents compared with the *B. cinerea* inoculated leaves about 1.6, 1.4, and 1.3 fold, respectively. On the contrary, reduction of photosynthetic pigment contents caused by the pathogen was folded by fungicide treatment. However, BABA + fungicide combination slightly reversed the negative effects on pigment contents related to the patogen infected and fungicide treated leaves.

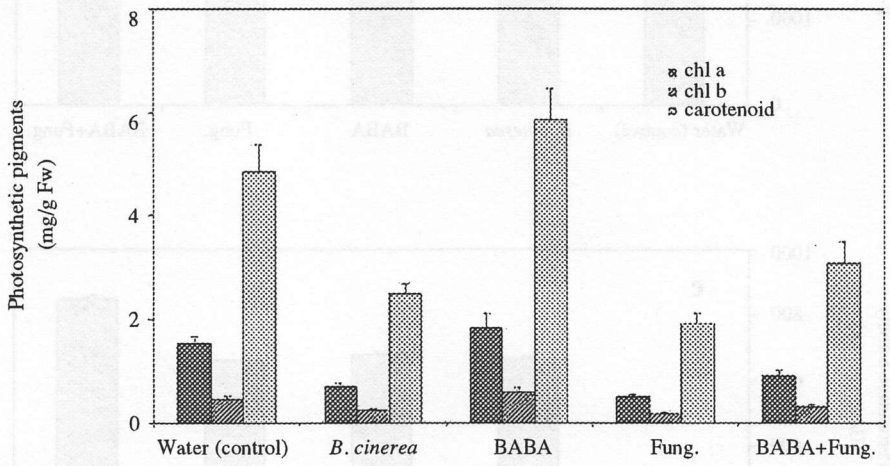

Figure 2. Photosynthetic pigment contents (mg/g) of control and treated leaves of pepper. Vertical lines on the graph denote the SE.

DISCUSSION

The data presented in this paper demonstrate that BABA protects pepper plants from infection by the necrotrophic fungal parasite *Botrytis cinerea*. Similar enhanced disease resistance induced by BABA was shown in pepper against *Pseudomaonas*

coscodes (Hong et al., 1999) and *Phytophthora capsici* (Hwang et al., 1997). This study confirms that the compound induces different levels of protection depending on the treatment combinations (single or combination with fungicide). When applied as single inducer, BABA gave moderate levels of protection (about 59.3%), whereas when applied in combination with fungicide (fenhexamide), it provided higher levels of control (about 82.2%) against *B. cinerea*. Supporting our data, Cohen (2001a) described that the synergistic interactions between BABA and fungicides, reporting, BABA-propicanozole combinations for reducing mildew infection in wheat or BABA-metalaxyl mixture for control of *Peronospora tabacina* infection in tobacco plants. Recently, Baider and Cohen (2003) was reported synergistic interactions between fungucide mancozeb and BABA for effective control of late blight in potato and tomato and downy mildew in cucumber.

In the present study, BABA has potentiated the leaf tissue to activate its pathogen defense mechanisms in response to *B. cinerea* by increasing chitinase and glucanase activity. Several reports indicated that BABA-activated PR protein accumulation, such as PR-1a, chitinase and β-1,3-glucanase proteins in pepper, tomato and citrus (Cohen and Gisi, 1994, Cohen et al., 1994; Hwang et al., 1997; Porat et al., 2003). It may be suggested that BABA-induced PR proteins were accumulated systemically in pepper, because obtained results were measured 14 days after treatment with BABA. Our data was coincides with the report of Hwang et al., (1997) that they both mention PR proteins were accumulated after a foliar spray of BABA. Close relationships were found between BABA-induced resistance and the activity of peroxidase. Increases in peroxidase activity often associated with a progressive incorporation of phenolic compounds with in the cell wall during incompatible plant-microbe/elicitor interactions. The association between resistance induced by BABA and enhanced enzyme activity of peroxidase was apparent in our data which was relevant to the results of Siegrist and Muhlenbeck (2001). They reported that BABA-induced cell death in lesions was associated with rapid generation of the reactive oxygen species superoxide, H_2O_2 and, consequently lipid peroxidation in pathosystems.

All enhancements of enzyme activities (chitinase, β-1,3-glucanase and peroxidase) were in accordance with the results of Cohen and Gisi (1994) mentioning for tomato plants that enhancing PR protein accumulation, BABA alters cell wall structure or metabolism so making the tissues more resistant to fungal enzyme attack. Moreover, the results from BABA + fungicide combination treated pepper plants show the highest enzyme activities in three of enzymes paralleled with infection control when compared with single BABA and fungicide treatments. These data also support the results of Cohen and Gisi (1994).

Although Cohen (2002) reported that BABA did not impair the photosynthesis by measuring sugar levels in tomato, the presented results show that the content of

photosynthetic pigments and root heights were elavated in BABA-treated pepper plants. Additionally, our results indicating an increase in pigments may be an indicator of the enhancement of the photosynhetic activity and increase in dry matter. This suggestion was supported by those of Shailasree et al., 2001 reporting BABA treated seeds of pearl millet were taller, had a larger leaf area and heavier dry weight than the control plants after 30 days of treatment. Moreover, BABA + fungicide treatment to leaves prevented chlorosis caused by fungicide (data not shown).

The data presented in this paper concerning the efficacy of BABA in field against natural attack by grey mould, makes BABA an attractive compound for practical agronomic use for *Botrytis cinerea* in pepper. Effect of BABA on inducing disease resistance is not only associated with the enhancement of the activity of PR proteins but also the recovery of the negative effects of fungicide on the pigment contents and growth and development of plant when used in a combination with fungicide.

ÖZET

BİBERDE *BOTRYTIS CINEREA*'YA DİRENCİN β-AMİNOBUTİRİK ASİT İLE UYARILMASI

Biber fideleri kullanılarak, proteinik olmayan bir aminoasit DL-beta-amino-n-butirik asitin (BABA), gri küf hastalığı etmeni *Botrytis cinerea*'ya karşı direnci uyarmadaki etkinliği araştırılmıştır. Yapraklara püskürtülen BABA'nın gri küf enfeksiyonunu %59.3 oranında kontrol edebildiği bulunmuştur. Ayrıca, BABA ve ardışık olarak uygulanan fungisitin (fenhexamide) sinerjik etkileşimi, BABA ve fungisitin tek başlarına uygulamalarına göre %82.2 düzeyine varan daha yüksek bir koruma sağlamıştır. BABA uygulanmış bitkilerde artan direnç, PR proteinleri kitinaz (PR-3), β-1,3 glukanaz (PR-2) and peroksidaz aktivitesindeki önemli artışla birlikte oluşmuştur. PR proteinleri ve proksidaz birikiminin, BABA'nın muhtemel etki şeklinde temel bir rol oynama olasılığı yüksektir. Hastalık direncinin uyarmasının yanı sıra, BABA fotosentetik pigmentlerin (klorofil a, klorofil b and karotenoidler) içeriğinde ve kök boyunda önemli artışa neden olmuştur.

Anahtar Kelimeler: Hastalık direnci, β-Aminobütirik asit, Biber, *Botrytis cinerea*

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