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First Report of Molecular Identification of Apple Viruses in Turkey

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Apple is one of the most important fruit crops in Turkey with the production of 2.500.000 tons (Anonymous, 2003). Despite of the importance of apple production, certification programme in apple have not been established yet and only a few researches have been performed on virus and virus-like diseases of apples in Turkey (Özkan and Kurçman, 1976; Nogay et al., 2001). Economically important common virus diseases of apple trees are *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple mosaic virus* (ApMV) and *Apple stem grooving virus* (ASGV). In the case of infection with above viruses, significant yield reduction on the trees (up to 60%) was possible, especially on mixed infections occurring frequently (Campell, 1963; Zahn, 1996). In order to certificate apple trees, the plants have to be tested for these four distinct viruses along with other plant pathogens (EPPO, 1999). Since, main apple viruses except ApMV are present symptomless on infected apple trees, testing of mother plants is very important for preventing virus spread (Figure 1). Applying reliable, fast and inexpensive methods are essential for routine diagnosis. Therefore, ELISA and RT-PCR were used as detection tools in this study to test apple samples, provided mainly from East Mediterranean Region of Turkey.

Plant samples were collected from Adana, Osmaniye and İçel provinces during the early spring of 2004. Leaves and shoots were tested by ELISA for ApMV, ASGV and ACLSV but not for ASPV because no antisera is available commercially for this virus (Nemchinov and Hadidi, 1998). Among 158 apple tree samples, 22 of them were detected as infected by ASGV, 38 samples infected by ACLSV, 16 samples infected by ApMV and 4 samples mix-infected by ASGV+ACLSV according to ELISA results. Randomly selected 80 samples some of which were detected positive for ASGV and ACLSV by ELISA, were tested for the all viruses by RT-PCR using Henegariu et al. (1997) method, except ApMV. All ELISA positive samples of ASGV and ACLSV were also found positive by RT-PCR as expected. On the other hand, the samples, which resulted very weak positive reaction or negative reaction by ELISA, were detected that 4 of them infected by ASGV and 21 of them infected by ACLSV according to RT-PCR. Beside this, 8 apple trees were found infected with ASPV by RT-PCR (Figure 2).

FIRST REPORT OF MOLECULAR IDENTIFICATION OF APPLE VIRUSES IN TURKEY

This research showed that apple virus diseases were very common on especially symptomless trees in the East Mediterranean Region of Turkey. To our knowledge, this is the first report on incidence and molecular identification of apple viruses in Turkey.



Figure 1. Early spring symptoms of Apple Mosaic Virus (ApMV) disease on Granny Smith apple variety in Gülnar-İçel.

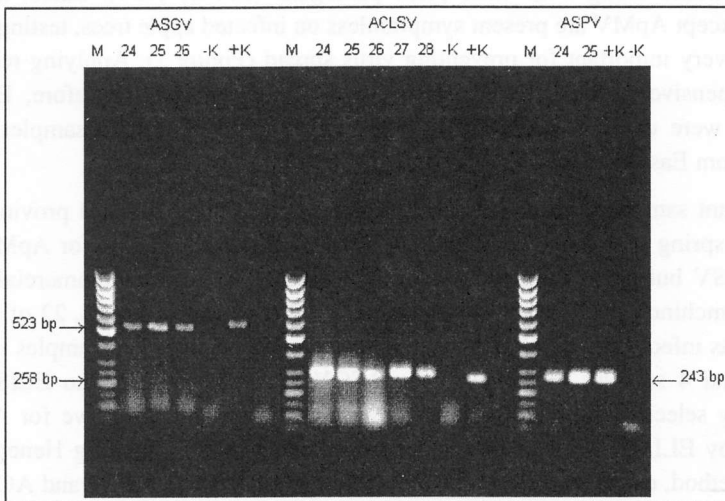


Figure 2. Representative results for the detection of *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV) by RT-PCR. M: Size marker; Lanes 24, 25, 26: Apple tree samples from Pozanti-Adana; +K: Positive control sample for ASGV, ACLSV and ASPV; -K: Water control.

ÖZET

TÜRKİYE'DE ELMA VİRÜSLERİNİN MOLEKÜLER TANILANMASI İLE İLGİLİ İLK RAPOR

Elma 2.500.000 ton üretimle Türkiye'de yetiştirilen en önemli meyvelerden biridir (Anonim, 2003). Elma üretimi ülkemizde bu kadar önemli olmasına karşın virüs ve virüs benzeri hastalıklarla ilgili çok az sayıda çalışma yapılmıştır ve henüz yürürlükte olan bir sertifikasyon programı da bulunmamaktadır (Özkan ve Kurçman, 1976; Nogay ve ark., 2001). Elma ağaçlarının ekonomik olarak önemli ve yaygın virüsleri Elma klorotik yaprak leke virüsü (ACLSV), Elma gövde çukurlaşma virüsü (ASPV), Elma mozaik virüsü (ApMV) ve Elma gövde oluklaşma virüsü (ASGV)'dür. Bu virüsler özellikle karışık enfeksiyon durumunda bulduklarında %60'a varan oranlarda verim azalmasına neden olmaktadır (Campbell, 1963; Zahn, 1996). Avrupa standartlarına göre elmalarda sertifikalı fidan üretimi için bitkilerin diğer patojenlerin yanı sıra bu dört virüse karşı da test edilmesi zorunludur (EPPO, 1999). Elma mozaik virüsü dışındaki diğer üç virüs elma ağaçlarında simptomsuz olarak bulunduğu için virüslerin yayılmasını önleme açısından anaç parsellerin test edilmesi son derece önemlidir (Şekil 1). Bu bitkilerin düzenli test edilmesi için de güvenilir, hızlı ve pahalı olmayan yöntemlerin tercih edilmesi gereklidir. Bundan dolayı bu çalışmada özellikle Doğu Akdeniz Bölgesi'nden toplanan örnekleri test etmek amacıyla ELISA ve RT-PCR yöntemleri kullanılmıştır.

Adana, Osmaniye ve İçel'den 2004 yılının erken ilkbahar döneminde bitki örnekleri toplanmıştır. Bu örnekler ApMV, ASGV ve ACLSV için ELISA ile test edilmiş ancak ASPV için henüz üretilmiş ticari antisera bulunmadığı için bu virüs ELISA ile test edilememiştir (Nemchinov ve Hadidi, 1998). Toplam 158 örnek arasından, ELISA sonuçlarına göre 22 tanesi ASGV, 38 tanesi ACLSV, 16 tanesi ApMV ve 4 tanesi de ASGV+ACLSV ile karışık enfeksiyon şeklinde enfekteli bulunmuştur. Bu örneklerden tesadüfi olarak seçilen ve bazıları ASGV ve ACLSV için ELISA pozitif bulunan 80 örnek, ApMV dışındaki üç virüs için Henegariu ve ark. (1997) metodu kullanılarak RT-PCR ile de test edilmiştir. ASGV ve ACLSV ile ELISA yönteminde pozitif bulunan tüm örnekler beklediği üzere RT-PCR ile de pozitif sonuç vermiştir. Bununla birlikte ELISA ile negatif bulunan veya çok zayıf pozitif sonuç vererek şüpheli durumda değerlendirilen örneklerden ASGV için 4, ACLSV için ise 21 örnek RT-PCR ile pozitif bulunurken ayrıca 8 örnek de ASPV ile enfekteli olarak saptanmıştır (Şekil 2).

Bu çalışma ülkemizin Doğu Akdeniz Bölgesi'nde özellikle simptomsuz ağaçlarda elma virüslerinin oldukça yaygın olduğunu göstermiştir. Bilgilerimiz doğrultusunda bu çalışma Türkiye'de elma virüslerinin yaygınlığının saptanması ve moleküler yöntemlerle tanılanmasına yönelik ilk kayıttır.

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Fungitoxic Effects of Hydrosols From Several Spices Against Some Phytopathogenic Fungi

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ABSTRACT

Fungitoxic effects of oregano (*Origanum vulgare* L.), fennel (*Foeniculum vulgare* L.), sea fennel (*Crithmum maritimum* L.), zahter (*Thymbra spicata*) and sage (*Salvia aucheri*) hydrosols on phytopathogenic fungi such as *Rhizoctonia solani* Kühn., *Fusarium oxysporum* f.sp. *tulipae* Synder & Hansen, *Botrytis cinerea* Pers. and *Alternaria citri* Ellis & Pierce were determined in vitro. All utilized doses of *Origanum* hydrosols showed fungicidal effect by inhibiting mycelium growth with an excellent rate (100%) of all tested fungi. While all doses of zahter hydrosol showed fungistatic effect on all the test fungi, only 15% dose of it completely inhibited the mycelial growth at *R. solani*. The fungistatic effect decreased gradually towards the end of incubation. *T. spicata* was the most effective on *R. solani* and *B. cinerea*. The fennel hydrosol (5%) stimulated the mycelial growth of *A. citri*. The increasing doses of this hydrosols showed more fungistatic activity against the fungi except *B. cinerea*. Sea fennel was more effective on *R. solani* and *B. cinerea* compared with *F. oxysporum* f.sp. *tulipae* and *A. citri*. Sage hydrosol showed no fungitoxic effect on *R. solani* and *F. oxysporum* f.sp. *tulipae*. But its increased doses were partially effective on *B. cinerea* and *A. citri*.

Key words: Fungitoxic effect, phytopathogenic fungi, spice hydrosols

INTRODUCTION

The excessive and indiscriminate use of organic pesticides has resulted in ill effects on soil health, health hazards to humans, toxicity to useful non-targeted animals and environmental pollution (Roy and Dureja, 1998). Therefore, alternatives to synthetic pesticides are needed from microbial and plant sources. Effective phytochemicals are expected to be far more advantageous than synthetic pesticides, as they are easily decomposable, not environmental pollutants and possess no residual or phytotoxic properties (Tewari 1990; Rao 1990; Badiei et al., 1996). The importance of spices and their derivatives (extract, essential oils, decoctions, hydrosols) in crop protection is being increasingly recognized under the concept integrated pest management (IPM). Under this concept, all possible modes of plant disease control methods should be integrated to minimize the excessive use of synthetic pesticides.

FUNGITOXIC EFFECTS OF HYDROSOLS FROM SEVERAL SPICES AGAINST SOME PHYTOPATHOGENIC FUNGI

The antimicrobial effects of spices and their derivatives have been reported several times (Deans and Svoboda, 1989; Bowers and Locke, 2000; Özcan and Boyraz, 2000; Sağdıç and Özcan, 2003). The leaves of *Labiatae* plants, sage, thyme, oregano and savory are used in meat, fish and food products since many years. It is known that various spices and herbs show antimicrobial activity. Spices and derivatives are being used in stored foods as a preservative against microbial contamination due to their activity. The inhibitory effects of some spices and herbs against mycotoxin producing fungi were determined in previous studies (Azzouz and Bullerman, 1982; Wright et al., 2000). It was reported that many plants and plant products have inhibitory effects on pests (Grange and Ahmed, 1988; Grayer and Harborne, 1994).

Although, most of the reports on natural products in agricultural areas are about insects, there are important reports revealing that plant extracts and essential oils exhibit antimicrobial activity against food and cereal store fungi, leaf pathogens and soilborne fungi (Kishore et al., 1989; Thomson, 1989; Muller-Riebau et al., 1995; Tewari 1995; Passini et al., 1997; Wilson et al., 1997; Bowers and Locke, 2000).

There are some studies on the determination of inhibitors in plants and using these inhibitors against detrimental organisms and microorganisms by artificial synthesis (Bhowmick and Chaudhary, 1982; Weltzien and Ketterer, 1986; Alice and Rao, 1987; Egger, 1987).

Although, more studies on antifungal properties of spice derivatives such as essential oil and extract against phytopathogenic fungi were conducted in last decade; there are few studies on antimicrobial properties of spice hydrosols (Özcan and Boyraz, 2000; Sağdıç and Özcan, 2003).

The aim of this study was to investigate the fungitoxic activity of hydrosols from (oregano, fennel, sea fennel, zahter, sage) against *Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *tulipae*, *Botrytis cinerea* and *Alternaria citri* in vitro.

MATERIALS and METHODS

Plant material

The spice species used were collected from different localities of Turkey in 2002 and dried at room temperature. They were identified at the Biology Department of Selçuk University (Table 1).

Table 1. Source of Hydrosols

Plant species	Plant family	Plant parts used
<i>Origanum vulgare</i> L. (Oregano)	Labiatae	Leave+flower
<i>Foeniculum vulgare</i> L. (Fennel)	Umbelliferae	Fruit
<i>Crithmum maritimum</i> L. (Sea fennel)	Umbelliferae	Leave+brunch
<i>Thymbra spicata</i> L. (Zahter)	Labiatae	Leave+flower
<i>Salvia aucheri</i> L. (Sage)	Labiatae	Leave+flower

Test Fungi

The test fungi (Table 2) were obtained from the collections of Plant Protection Department, Faculty of Agriculture, Selcuk University.

Table 2. The origins of the test fungi

Fungi	Origin
<i>Rhizoctonia solani</i> Kühn.	Potato tuber (Nevşehir-Derinkuyu)
<i>Fusarium oxysporum</i> f.sp. <i>tulipae</i> Snyder&Hansen	Tulip bulb (Konya-Çumra)
<i>Alternaria citri</i> Ellis&Pierce	Lemon fruit (İçel-Erdemli)
<i>Botrytis cinerea</i> Pers.	Tomato fruit (Muğla - Fethiye)

Culture Media

Test fungi were grown on potato dextrose agar (200 g diced potato, 20 g dextrose, 15 g agar-agar, 1000 ml distilled water). Czapek Dox Agar (30 g sucrose, 3 g sodium nitrate, 0.5 g magnesium sulphate, 1 g potassium hydrogen phosphate, 13 g agar, 1000 ml distilled water) was used for the detection of fungitoxic effects.

Preparation of hydrosols

A 50 g of each spice species was ground in an omnimixer. The hydrosols were obtained by using an hydrodistillation apparatus with 500 ml water (1:10;w/v) for 1h at 100°C. Then, its oil was removed from mixed solution by separation funnel. After hydrosols were filtered, and kept in steril bottles (500 ml) under refrigerated conditions until use.

Detection of fungitoxic effects of hydrosols

150 ml culture medium (Czapex Dox Agar) was prepared for testing the effects of each spice hydrosols and doses. After sterilization at 121°C for 20 min. in an autoclave, 5%, 10%, 15% doses of hydrosols were added into agar medium; shaken thoroughly and poured into Petri dishes. After overnight in labour conditions, the plates were inoculated with fungal discs (5 mm ϕ) taken from the colonies. Petri dishes were incubated at 24-25°C. Assays were carried out in two duplicates

Evaluation of Fungitoxic effects

In order to evaluate the inhibition of mycelial growth, colonial diameters were measured after incubation. The inhibition rates were calculated according to a formula by Deans and Svoboda (1990):

$$I = (C-P/C) \times 100$$

I : Inhibition (%)

C : Colonial diameter on control plates (mm)

P : Colonial diameter on treated plates (mm)

FUNGITOXIC EFFECTS OF HYDROSOLS FROM SEVERAL SPICES AGAINST SOME PHYTOPATHOGENIC FUNGI

Moreover, mycelial discs showing no growth were transferred to hydrosol free PDA plates. After following incubation for a week, any further growth indicated a fungistatic effect; if no development was noted, the effect was appreciated as fungicidal.

RESULTS and DISCUSSION

Fungitoxic effects of hydrosols from 5 spice species against four phytopathogenic fungi are given in Table 3. A complete inhibition was obtained from 3 different doses of oregano hydrosols against mycelial growth of all fungi. In all doses of oregano hydrosols 100% fungicidal effect was observed.

Fennel hydrosol showed various degrees of toxic effects against the fungi tested, while 5% dose of fennel hydrosol stimulated mycelial growth of *A. citri*. However, 10% and 15% doses of this hydrosol possessed 14.70% maximum and 3% minimum inhibition. Increasing levels of hydrosol doses, caused higher inhibition on the mycelial growth of *R. solani* and *F. oxysporum* f.sp. *tulipae* except *B. cinerea*. But, 5% dose of fennel hydrosol was seen to be higher fungitoxic on *B. cinerea* than 10% and 15% doses.

Hydrosol of sea fennel showed stronger fungitoxic activity against *R. solani* and *B. cinerea* and fairly low activity against *F. oxysporum* f.sp. *tulipae* and *A. citri*. During incubation, any doses of sea fennel showed fungitoxic activity against *A. citri* and *F. oxysporum* f.sp. *tulipae* after the five and six days, respectively.

As it can be clearly seen in Table 3 zahter hydrosols exhibited variable degree of fungitoxic activity against all the fungi tested.

Hydrosol of zahter was the second fungitoxic effective hydrosol coming after oregano hydrosol, among the all hydrosol used in the assay. Hydrosol of zahter showed the highest fungitoxic activity against *R. solani*. 15% dose of zahter hydrosol showed 100% fungi- toxic effect against *R. solani*. This complete effect was observed against *B. cinerea* at the same dose too, but this effect decreased 75.48% through the end of incubation period. While 5% and 10% doses of zahter hydrosol exhibited fungistatic activity, 15% dose, exhibited fungicidal activity.

Any doses of sage hydrosol exhibited fungitoxic activity against *R. solani*; contrary, it stimulated mycelial growth of this fungus. Similar results were obtained with the 5% dose of sage hydrosol against *F. oxysporum* f.sp. *tulipae* and *A. citri*. By 10% and 15% doses, mycelial growth of *F. oxysporum* f.sp. *tulipae* was not affected, but *A. citri* was inhibited moderately.

Consequently, the hydrosol of oregano caused a complete inhibition (100%) on the mycelial growth of all phytopathogenic fungi tested.

Table 3. Fungitoxic effects of hydrosols at different concentrations in culture medium

Test Fungi	Inhibition in Colonial Growth (%)																
	Hydrosols in Culture Medium (%)																
	<i>Origanum vulgare</i> L.			<i>Foeniculum vulgare</i> L.			<i>Crithmum maritimum</i> L.			<i>Thymbra spicata</i> L.			<i>Salvia aucheri</i> L.				
Days	5	10	15	5	10	15	5	10	15	5	10	15	5	10	15		
<i>Rhizoctonia solani</i>	3	100.00	100.00	100.00	28.37	32.43	38.67	15.09	23.21	31.13	64.93	100.00	100.00	S	S	-	
	4	100.00	100.00	100.00	25.55	31.28	36.66	14.00	22.64	29.40	63.39	100.00	100.00	S	S	S	
	5	100.00	100.00	100.00	23.05	30.41	35.45	11.68	21.30	28.54	52.22	91.11	100.00	S	S	S	
	6	100.00	100.00	100.00	21.62	27.45	29.41	6.25	20.77	24.47	42.71	87.10	100.00	S	S	S	
	7	100.00	100.00	100.00	20.35	25.42	27.35	5.4	16.12	20.37	26.22	79.09	100.00	S	S	S	
	<i>Fusarium oxysporum</i> f. sp. <i>tulipae</i>	3	100.00	100.00	100.00	6.45	19.35	48.97	6.01	7.30	7.40	16.66	52.77	69.44	S	S	-
		4	100.00	100.00	100.00	14.28	34.69	35.48	4.08	4.08	4.08	14.44	50.79	69.38	S	-	-
5		100.00	100.00	100.00	14.51	30.64	32.25	1.58	1.56	1.54	12.69	48.97	68.25	S	-	-	
6		100.00	100.00	100.00	13.50	27.4	30.51	**	-	-	10.20	46.79	67.16	S	-	-	
7		100.00	100.00	100.00	11.54	24.37	26.45	-	-	-	9.10	44.44	66.66	S	-	-	
<i>Botrytis cinerea</i>		3	100.00	100.00	100.00	35.41	33.85	29.13	20.93	24.74	31.39	62.79	86.04	100.00	13.95	22.09	25.58
		4	100.00	100.00	100.00	29.83	31.10	20.54	18.22	21.66	28.14	55.55	80.00	85.55	-	12.22	16.52
	5	100.00	100.00	100.00	25.23	30.00	19.62	14.21	18.10	24.06	46.22	74.2	82.07	-	6.66	7.12	
	6	100.00	100.00	100.00	24.65	28.76	15.55	10.05	14.07	18.06	42.06	70.01	78.04	-	-	-	
	7	100.00	100.00	100.00	24.44	27.65	14.21	8.62	12.21	16.32	38.47	67.50	75.48	-	-	-	
	<i>Alternaria citri</i>	3	100.00	100.00	100.00	S*	8.82	14.70	2.77	5.55	5.55	33.33	53.33	72.22	S	18.5	18.10
		4	100.00	100.00	100.00	S	7.13	12.5	2.17	2.17	3.32	31.76	52.17	69.56	S	12.30	12.57
5		100.00	100.00	100.00	S	5.00	12.19	-	-	-	30.33	51.85	68.51	S	9.64	9.93	
6		100.00	100.00	100.00	S	4.16	12.00	-	-	-	27.77	46.38	64.46	S	5.5	5.8	
7		100.00	100.00	100.00	S	3.00	10.62	-	-	-	24.32	43.64	60.54	S	4.3	4.6	

* stimulation
** no inhibition

FUNGITOXIC EFFECTS OF HYDROSOLS FROM SEVERAL SPICES AGAINST SOME PHYTOPATHOGENIC FUNGI

The analysis showed that fennel and sea fennel hydrosols exhibited certain degrees of fungistatic activity depending on the doses. But, sage hydrosol possessed no inhibitive effect on the mycelial growth of some fungi tested, contrary, it stimulated the mycelial growth of *R. solani* and *F. oxysporum* f.sp. *tulipae*. Additionally, the hydrosol of sage partially inhibited the mycelial growth of *B. cinerea* and *A. citri*, depending on the increasing doses.

Several studies have conducted on the antimicrobial properties of herbs,spices and their derivatives such as essential oils, extracts and decoctions (Farag et al., 1989; Ushiki et al., 1996; Hammer et al., 1999; Bowers and Locke, 2000; Dorman and Deans, 2000; Özcan and Boyraz, 2000; In addition, it is know that the compositions of hydrosols and their antimicrobial effects depend on the plant species and regional conditions. Some authors established a relationship between the chemical structures of the most abundant compounds in the tested essential oils and the antimicrobial activity (Deans and Svoboda, 1990; Caccioni et al., 1998). Vigorous plant tissues contain too much natural antimicrobial constituents and these compounds defense plants against various diseases (Fawcet and Spencer, 1970).

In an earlier work on the fungitoxic properties of some plant species growing in the south Anatolia, *Thymbra spicata*, *Satureja thymbra*, *Salvia fruticosa*, *Laurus nobilis*, *Mentha pulegium*, *Inula viscosa*, *Pimpinella anisum*, *Eucalyptus camaldulensis* and *Origanum minutiflorum* were found to have various fungitoxic compounds such as gamma-terpinen, p-cymenen, thymol, carvacrol, 1-8 cineole, pulegone and anethole which inhibit especially some soilborne fungi like, *F. moniliforme*, *R. solani*, *Sclerotinia sclerotiorum* and *Phytophthora capsici* (Muller-Riebau et al., 1995). *Origanum syriacum* which contains carvacrol and thymol were reported to be effective on *Aspergillus niger*, *F. oxysporum* and *Penicillium* spp. (Daouk et al., 1995).

Our observations indicate that oregano and zahter hydrosols are a potent antifungal agent with broad spectrum activity with possible potential for the control of fungal infections in plants as well as that of post- harvest spoilage of many crops/ crop products. Further studies have to be done in plant model systems to evaluate the efficacy of oregano and zahter hydrosols under pot and field conditions against induced fungal infections so that its importance as an alternative to synthetic.

ÖZET

BAZI BAHARAT HİDROSOLLERİNİN FİTOPATOJENİK FUNGUSLAR ÜZERİNDE TOKSİK ETKİLERİ

Mercanköşk (*Origanum vulgare* L.), rezene (*Foeniculum vulgare* L) deniz rezenesi (*Crithmum maritimum* L.) kekik (*Thymbra spicata* L.) ve adaçayı (*Salvia aucheri* L.) hidrosollerinin *Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *tulipae*, *Botrytis cinerea*

ve *Alternaria citri* gibi fitopatojenik funguslar üzerindeki fungitoksik etkileri in vitro'da saptanmıştır. Mercanköşk hidrosolünün kullanılan tüm dozları, denenen tüm fungusların miseliyal gelişimini %100 engelleyen fungisidal etki göstermiştir. Kekik hidrosolünün yalnızca %15'lik dozu *R. solani*'nin miseliyal gelişimini %100 engellerken, diğer funguslara tüm dozlarında fungistatik etki görülmüştür. İnkubasyonun başlangıcındaki yüksek fungistatik etki inkubasyonun sonuna doğru yavaş yavaş azalmıştır. Kekik hidrosolünden en çok *R. solani* ve *B. cinerea*'nin misel gelişimi etkilenmiştir. Rezene hidrosolünün %5'lik dozu *A. citri*'nin miseliyal gelişimini teşvik ederken, diğer muamelelerde test edilen funguslara karşı değişik derecelerde fungistatik aktivite sergilemiştir. Bu bitkinin artan hidrosol dozları *B. cinerea* dışındaki funguslara karşı daha yüksek fungistatik aktivite göstermiştir. Deniz Rezenesi hidrosolü *R. solani* ve *B. cinerea*'ya, *F. oxysporum* f.sp. *tulipae* ve *A. citri*'den daha fazla etkili olmuştur. Adaçayı hidrosolü *R. solani* ve *F. oxysporum* f.sp. *tulipae*'ye karşı fungitoksik etki göstermemiştir. Fakat bu bitkinin artan hidrosol dozları *B. cinerea* ve *A. citri*'ye nisbi olarak etkili olmuştur.

Anahtar Kelimeler: Baharat hidrosolleri, fitopatojenik funguslar, fungitoksik etki

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Applicability of TLC in Pesticide Residue Analysis Using Bioassay with Fungi Spores

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ABSTRACT

Thin Layer Chromatography (TLC) is widely used in most analytical laboratories for the separation of soluble mixtures containing known and unknown compounds and for the qualitative identification and quantitative determination of known compounds. This study was undertaken to determine the retention factor (R_f), relative retention factor (RR_f), and minimum detectable quantities (MDQ) of some fungicides (captan, fenarimol and prochloraz) with fungi spore inhibition TLC detection method. The verification of MDQ's of the compounds was performed in the presence of cleaned-up wheat-grain extract. The limit of detection (LOD) of fungicides were calculated.

Key words: TLC-detection methods, fungi bioassay

INTRODUCTION

TLC has grown in recent years and is now widely accepted as a rapid and efficient detection technique. Although most advances in pesticide analysis during the past few years have taken place in the field of Gas Chromatography (GC) and High Pressure Liquid Chromatography (HPLC); TLC has retained its status as a valid and simple method for the qualitative and quantitative analysis of pesticide residues and their metabolites. In addition to this some nonvolatile or labile pesticides can not be analysed by GC (Rathore and Sharma, 1992; Rathore and Begum, 1993).

The method developed is intended for laboratories where the irregular supply electricity, lack of service or the limited budget do not allow the continuous use of GC and HPLC techniques. The TLC detection may also be used together with the GC or HPLC detection for confirmation of the residues in other laboratories (Ambrus et al., 1996).

TLC analysis requires reproducible R_f . To ensure the reproducibility of the R_f values and the required efficiency, the conditions have to be controlled. The main parameters influencing reproducibility of HR_f ($R_f \times 100$) are type and quality of the

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adsorbent, activity of the layer thickness, saturation of the chamber, humidity of the air, temperature, and elution solvent (Lantos, 1998). The use of the so-called marker compounds have proven to be very satisfactory for this purpose. A marker compound was selected for each detecting reagent. The MDQ and R_f values of marker compounds referring to the different detection methods were determined and tabulated by Füzesi et al. (1998).

The MDQ of the marker compound should be spotted on each plate on which qualitative and 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 relatively stable in standard solutions; be sensitive for the detection conditions (not appearing on the plate if the conditions are not optimal); have reproducible R_f values (Ambrus et al., 1981).

It was tested the applicability of TLC in pesticide residue analysis, using the following general modes of detection: o-tolidine plus KI, $AgNO_3$ plus UV radiation, photosynthesis inhibition, bioassay with fungi spores, enzyme inhibition (Füzesi et al., 1998). Biological test procedures are useful for the specific detection of compounds with a certain physiological activity. For example fungicide compound zones can be detected by spraying the plate with the suspension of fungi, incubating, and observing zones where there is no fungal growth (Lantos, 1998).

The fungi spore inhibition method provides the best selectivity and plant extracts usually do not interfere. It detects only fungicides at residue level (Ambrus et al., 1996).

The purpose of the study reported here was to investigate the possibilities of applying fungi spore (*Aspergillus niger*) inhibition TLC detection method in combination with the widely used ethyl acetate extraction and Gel Permeation Chromatography (GPC) SX-3 clean-up method for providing an alternative cost effective analytical procedure for screening pesticide residues in plant commodities using grains.

MATERIALS and METHODS

Materials

Equipment

General laboratory equipment and TLC basic set were used in this study. For clean-up wheat extracts GPC apparatus were used.

Pesticides

Captan, fenarimol, prochloraz active ingredients were used as marker compounds referring to the fungi spore (*Aspergillus niger*) inhibition (FAN) detection methods

(Ambrus et al., 1981; Ambrus et al., 1996). The pesticide standards obtained from Dr. Ehrenstorfer Laboratories GmbH, Germany via the International Atomic Energy Agency (IAEA), were used in the project.

Chemicals

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

Table 1. Tabulated MDQ and R_f values of marker compounds referring to the FAN method¹ (Füzesi et al., 1998)

Compound	MDQ	R _f
<u>Captan</u>	20 ng	0.630
Fenarimol	50 ng	0.476
Prochloraz	25 ng	0.314

¹ Development solvent: Ethyl acetate; Layer: Silica gel 60, 0.25 mm thickness

Reagents

Preparation of *Aspergillus niger* spore suspension: 1.5 g agar-agar boiled in 70 ml water, added 1.5 g glucose, 0.3 g KNO₃ and 1.3 g Malt extract. The mixture was cooled to 50 °C and kept the solution at that temperature. *A. niger* spores were rinsed from the culture by adding 30 ml bi-distilled (3 times with 10 ml) water and cautiously drawing with plastic or glass spatula.

The spore suspension contains required number of spores if its colour is dark grey. The suspension was mixed with agar-agar solution. The mixture was kept in a jar in 45 °C water bath without loss of activity. The suspension was filtered through 2-layer gauze (Ambrus et al., 1981 ; Ambrus et al., 1996).

Methods

Elution of pesticides on TLC plates

The silica gel 60 layer, 0.25 mm thickness (Merck 1.05721) was activated at 105 °C for 30 minutes and later put the desiccator for cooling. 20 µl extract and/or standard solutions were applied in uniform spots of about 4-6 mm on chromatographic plate 2 cm from bottom. It was spotted the mixture of marker compounds (Table 1) on each plate in different MDQ's (Table 2).

The R_f values were determined in developing tanks kept in water bath thermostated at 23 °C in order to reduce the effect of temperature variation in the laboratory. The eluent (ethyl acetate) was equilibrated with the vapour phase by inserting filter paper in the developing tank and waiting for minimum 30 minutes before the plates were placed 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 origin (Ambrus et al., 1996).

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Fungi spore (*Aspergillus niger*) inhibition (FAN) detection method

The extract and/or standard solutions were chromatographed on Silica gel 60 plate and dried the plate with gentle air stream after elution. The atomiser was warmed up by immersing it into 40 °C de-ionised water bath to avoid that the agar-agar cools down and the solid particles block the atomiser. The spore suspension of *A. niger* was sprayed the air-dried plate until the layer gets thoroughly wet but avoid run-off, and put it immediately for incubation. Instead of an incubator, simple way was introduced by Asi¹; a developing tank with ground glass lid can be very efficiently used. The tank was saturated with water in an oven at 37 °C. It was kept about 2 cm water in the tank. The upper 3 cm of the adsorbent layer was removed from the plate and inserted the cleaned part of it in the water and closed the lid and then the tank was placed for 48 hours in the oven. Inhibition zones of fungicides can be observed as bright spots in velvety background of growing fungus mycelia. Spots are more clearly visible if plates are observed in the right narrow angle of reflected light (Ambrus et al., 1996).

Calibration of GPC, extraction and clean up

Gel Permeation Chromatography column (200 mm x 10 mm glass column) was filled with 8 g Bio-Bead SX-3 gel as described in the manual (Anonymous,1998). The GPC column was calibrated by using ¹⁴C-chlorpyrifos at the flow rate of 1 ml/min. Total 30469 dpm ¹⁴C-chlorpyrifos was injected in 250 µl ethylacetate: cyclohexane (1:1) mixture solution to the column. The collected fractions were radioassayed by using 1550 Tri-Carb Liquid Scintillation Analyzer. Extraction of ground wheat and clean up procedure were performed as described by Füzesi et al. (1998).

RESULTS and DISCUSSION

Marker compounds results

Under the specific conditions described for FAN detection methods, the retention factors (R_f) were measured with 3 runs for each marker compound. The spotting format, based on different MDQ concentrations, for determining the R_f , and RR_f values of captan, fenarimol and prochloraz are given in Table 2.

The R_f , RR_f , and their CV's and MDQ's determined with FAN detection method are given in Table 3. To compare our data, tabulated R_f , RR_f and MDQ values by Füzesi et al. (1998), included in Table 3. The determined MDQ values in our laboratory are lower than Füzesi et al. (1998)'s findings. The R_f and RR_f values were in close agreement with the reported ones, except prochloraz. Since there is a lot of factors influencing the reproducibility of R_f (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.

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Table 2. The spotting format and determined R_f , RR_f values of the marker compounds with the FAN detection method (Average of 3 plate).

Spot No.	Applied MDQ eq. ^a	R_f of Fenarimol	R_f of Prochloraz	$R_{f_{ref}}^c$ (Rf of Captan)	RR_f of Fenarimol	RR_f of Prochloraz
1	5	0,486	0,282	0,653	0,745	0,433
2	2	0,481	0,282	0,655	0,735	0,431
3	1.5	0,479	0,284	0,657	0,729	0,431
4	1	0,479	0,282	0,656	0,731	0,430
5	0.75	0,479	0,284	0,656	0,731	0,433
6	0.5	<u>0,476^b</u>	<u>0,279</u>	<u>0,656</u>	0,726	0,426
7	0.5	0,476	0,281	0,658	0,724	0,427
8	0.75	0,479	0,281	0,656	0,731	0,428
9	1	0,479	0,284	0,657	0,729	0,431
10	1.5	0,478	0,285	0,659	0,725	0,432
11	2	0,478	0,282	0,657	0,726	0,429
Average R_f		0,479	0,282	0,656	0,730	0,430
Standard deviation:		0,003	0,002	0,002	0,005	0,002
Variance:		6,6E-06	2,9E-06	3,01E-06	3,05E-05	6,01E-06
Coefficient of variation:		0,005	0,006	0,002	0,007	0,006

^a MDQ is the value reported in the background technical document by Füzesi et al. (1998). 1 MDQ for captan, fenarimol and prochloraz are 20 ng, 50 ng and 25 ng, respectively.

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

^c $R_{f_{ref}} = R_f$ values of reference marker compound (captan)

Table 3. The R_f , RR_f and MDQ values of the compound determined with FAN method.

Compound	Reproducibility of R_f values ^a					Reproducibility of RR_f^b values		Reported ^c		MDQ, ng	
	1	2	3	Mean	CV (%)	Mean	CV (%)	R_f	RR_f	Reported ^c	Found
Captan	0.659	0.643	0.666	0.656	1.78	1	0	0.63	1	20	10
Fenarimol	0.461	0.478	0.498	0.479	3.86	0.730	3.63	0.476	0.756	50	25
Prochloraz	0.274	0.275	0.296	0.282	4.39	0.430	3.29	0.314	0.498	25	12.5

^a EtAc -precoated silica gel system

^b $RR_f = R_f / R_{f_{ref}}$ of reference compound Captan

^c Reported R_f , RR_f and MDQ by Füzesi et al. (1998).

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Calibration of GPC column

Although it is determined in the GPC Manual (Anonymous, 1998) that pesticide fractions comes through the column after the 10 ml, determining laboratory's own elution profile is very important. Figure 1 and Table 4 shows the elution profiles of ^{14}C -chlorpyrifos 94.35% of ^{14}C -chlorpyrifos came through the column in the 9-13 ml fractions at the 1 ml/min flow rate of eluent (EtAC: Cyclohexane 1:1). According to Füzesi et al. (1998) diazinon and triazophos comes through th column in the 9-16 ml fractions.

Figure 1. Distribution of ^{14}C -chlorpyrifos on Bio-Beads SX-3 gel with EtAC: Cyclohexane (1:1)

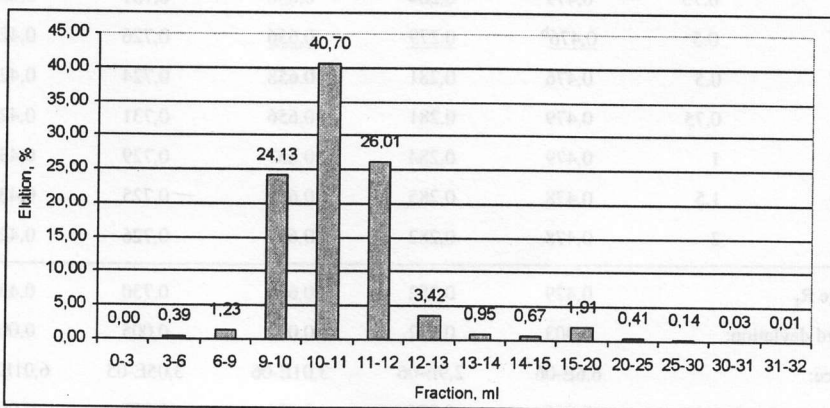


Table 4. Elution profile of ^{14}C -chlorpyrifos on Bio-Beads SX-3 gel with EtAC:Cyclohexane (1:1)

Eluent ml	Elution of ^{14}C -Chlorpyrifos	
	Count (dpm)*	Elution %
0-3	0	0.00
3-6	103	0.39
6-9	326	1.23
9-10	6376	24.13
10-11	10754	40.70
11-12	6873	26.01
12-13	903	3.42
13-14	250	0.95
14-15	178	0.67
15-20	504	1.91
20-25	108	0.41
25-30	38	0.14
30-31	7	0.03
31-32	2	0.01
Total	26422	100
Background	30	

* Count-Background dpm

Table 5. The verification data of MDQ values of the compounds with the FAN detection method in the presence of wheat extract (average of 3 plate)

Spot No.	Applied MDQ eq. ^a	R _f of Fenarimol	R _f of Prochloraz	R _{f,ref} d (R _f of Captan)	RR _f of Fenarimol	RR _f of Prochloraz
1	E ^b	-	-	-	-	-
2	E+MDQ	0.4725 ^c	0.2882	0.6607	0.7151	0.4362
3	E+1.5MDQ	0.4741	0.2909	0.6683	0.7094	0.4352
4	E+2MDQ	0.4625	0.2937	0.6682	0.6921	0.4395
5	E+3MDQ	0.477	0.2967	0.664	0.7184	0.4468
6	MDQ in solv.	0.5075	0.2923	0.6641	0.7642	0.4401
Average R _f		0.4787	0.2923	0.6650	0.7198	0.4396
Standard deviation:		0.01698	0.00316	0.0032	0.0268	0.0045
Variance:		0.00028	1E-05	1.04E-05	0.0007	2.07E-05
Coefficient of variation:		0,005	0,006	0,002	0,007	0,006

^a Found MDQ values in the laboratory; 1 MDQ for captan, fenarimol and prochloraz are 10 ng, 25 ng and 12,5 ng respectively.

^b Wheat sample extract; 10 mg sample eq./20 µl

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

d R_{f,ref} = R_f values of reference marker compound (captan)

Verification of MDQ of the active ingredients in the presence of wheat extract

The aim 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, the sample equivalent spotted onto the TLC plates should be reduced or further clean-up may be necessary to achieve the required sensitivity.

The verification tests were carried out in three replicates with the blank extracts of wheat after the GPC clean-up. The cleaned wheat extract was applied on the TLC plate and spotted over it with MDQ (in solvent). The verification of MDQ of captan, fenarimol and prochloraz in the presence of cleaned-up plant extract are summarized in Table 5. The spotting amount that could be spotted on to the TLC plate were found experimentally. It was found that, 10 mg sample equivalent applied in 20 µl did not affect the detectability of the compounds with the detection procedure. The LOD was calculated according to following equation.

$$\text{MDQ (ng)}$$

$$\text{LOD} = \frac{\text{MDQ (ng)}}{\text{M (mg)}} \text{ where M is the sample equivalent applied on the layer.}$$

$$\text{M (mg)}$$

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The calculated LOD values of fungicides are shown in Table 6.

Table 6. Verification of MDQ of the fungicides in the wheat extracts.

TLC Detection Method	Active Ingredients	MDQ, ng		LOD ^c , ng/mg Wheat extract
		Found ^a	Reported ^b	
FAN	Captan	10	20	1
	Fenarimol	25	50	2.5
	Prochloraz	12.5	25	1.25

^a Found MDQ values in the laboratory

^b Reported MDQ by Füzési et al. (1999)

^c LOD=MDQ (ng)/10 (mg)

ÖZET

FUNGUS SPORLARI BİOASSAY METODU İLE İNCE TABAKA KROMATOĞRAFİSİNİN PESTİSİT KALINTI ANALİZLERİNDE UYGULANMASI

İnce tabaka kromatografisi (TLC) birçok analitik laboratuvarlarda bir solusyon içindeki bilinen ve bilinmeyen bileşikleri ayırt etmekte yaygın olarak kullanılmaktadır. Bu çalışmada bazı fungusitlerin (captan, fenarimol ve prochloraz) fungus sporlarını engelleme metodu kullanarak TLC ile alıkonma faktörleri (R_f), relatif alıkonma faktörleri (RRf) ve en az tespit edilebilen miktarları (MDQ) belirlenmiştir. Temizlenmiş (clean-up) buğday ekstraktları TLC ye uygulanmış sonra da üzerine solvent içindeki MDQ lar spotlanarak bulunan MDQ değerleri doğrulanmıştır. Aynı zamanda bu fungusitlerin dedeksiyon limitleri (LOD) hesaplanmıştır.

Anahtar Kelimeler: TLC-dedeksiyon metotları, fungi biyoassay

ACKNOWLEDGEMENTS

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Occurrence of *Wheat streak mosaic virus* (WSMV) on cereals in Trakya Region in Turkey

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ABSTRACT

A virus disease has been occurred on some cereal species since 1999 in Trakya Region of Turkey. The Pathogen of this disease was identified as *Wheat streak mosaic virus* (WSMV). Severe leaf mosaic, necrosis of tillers, stunting and yield lost in infected winter wheats (*Triticum aestivum* L.) were the most characteristic symptoms of the pathogen. Ninety samples were collected from 54 infected fields in Trakya Region. Samples were tested by mechanical inoculation on eight indicator plant series. Ten leaf samples caused characteristic virus symptoms at least on two indicator species, and 27 samples gave symptoms only on one indicator plant.

Eleven polyclonal antibodies prepared against cereal viruses were used to virus diagnose by the DAS-ELISA tests. Among them, nine out of 90 samples revealed presence of WSMV in leaf samples of winter wheat. One of the wheat samples containing mechanically transmitted WSMV was employed for the preparation of grids of transmission electron microscopy (TEM). TEM studies revealed that flexible rod shape virions with a dimension of 15x750 nm were observed. According to results approached from biological tests, serological and electron microscopical investigations, this virus was identified as WSMV. So far, there were no data about the occurrence of WSMV in Trakya Region of Turkey.

Key words: *Wheat streak mosaic virus*, ELISA, virus, cereal

INTRODUCTION

Cereal production has been the most important farming type as Winter wheat (*Triticum aestivum* L.) being the major field crop in Turkey (Kün, 1988). Trakya Region however provides 7 % arable land to cereal production and realizes 13 % of yield in Turkey (Anonymous 2000), (Beşer et al., 2001). But Wiese (1987) listed 77 Pathogenic cereal diseases and many stress factors which reduce yield and quality of crop. Among them at least 12 different viruses were identified as pathogens of important diseases on

OCCURRENCE OF *Wheat streak mosaic* virus (WSMV) ON CEREALS IN TRAKYA REGION IN TURKEY

cereals in Asia and Europe. *Wheat streak mosaic* virus (WSMV) was described for the first time by Bremer (1971) in İzmir. Rabenstein et al. (2002) proved that Turkish isolates of WSMV taken from İzmir area and those American isolates of WSMV were identical genomically. This implies that WSMV was present in Turkey even in 19 century or before and transferred to the U.S.A by immigrants who carried red winter wheat seeds to new world. Brunt et al. (1996) described WSMV as mechanically transmissible virus containing flexible rods virions measured at least 13x700 nm and transmitted by nymphs and adults of *Aceria tulipae* (Eriophyidae) mite in field conditions. WSMV is found very destructive on winter wheat and diagnosed easily with high precision by using polyclonal or monoclonal antibodies (Montane et al., 1996). Beside *A. tulipae* WSMV can be transmitted by wheat curl mite *A. tosichella* with a persistent manner (Mahmood et al., 1997). Beside DAS-ELISA technique Mc Neil et al. (1996) proved that WSMV can be identified by employing reverse transcriptase-polymerase chain reaction (RT-PCR) methods.

The purpose of this study is to identify virus which has caused dwarfing, necrosis on tillers rosetting and reduction in yield of cereal crop in Trakya Region in Turkey since 1999.

MATERIALS and METHODS

Preliminary field inspections were conducted in Edirne, Kırklareli and Tekirdağ provinces of Trakya Region during March, April and May 2000, for the determination of cereal virus infection fields. During April and May, 2001 survey study was conducted and disease incidence was recorded and samples were collected from the infected fields in the locations displayed in Figure 1. Ninety samples each of them exhibiting symptoms and containing 500 gr leaf materials were collected, packed in tagged polyethylene bags listed in Table 1. They were kept in deep-freeze at -27 °C until they were used for biological, and serological tests and grid preparations for electron microscopy.

Mechanical Inoculation for Biological tests:

Eight indicator plants; Wheat: *Triticum aestivum* L., Barley: *Hordeum vulgare* L., Oat: *Avena sativa* L., Rye: *Secale cereale* L., Corn: *Zea mays* L., *Chenopodium quinoa* L., *Chenopodium amaranticolor* Costa & Reyn and *Poa pratensis* L. were obtained as healthy seedlings in greenhouse conditions 25 ± 5 °C temperature. Plant saps were obtained from the leaf samples by grinding leaves with a mortar and pestle at a 10:15 dilution (w:v) in 0.02 M phosphate buffer, pH: 7.2 according to modified method from Brakke (1958). Test plant seedling series were dusted with celite and were rubbed with sap, washed under tap water. Inoculated plants were maintained in green house conditions and controlled for characteristic symptom expression. At least two weeks after inoculation, positive and negative reactions of indicator test plants to viruses were recorded.

Serological Tests (DAS-ELISA)

Plant samples were homogenised using a Homex homogenizator with the addition of ELISA sample phosphate buffer solution at a ratio of 1:5 dilution (v:w) according to the method of Clark and Adams (1977) modified by Lister and Rochow (1979). Eleven antisera prepared against different cereal viruses were employed to the determination of the presence of different viruses. Antisera used are listed in Table 2. ELISA test kits were selected and obtained from Germany-Aschersleben Pflanzenzüchtung und Phytopathology Institut. Two ELISA test plates containing 96 wells were used for each antisera. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was employed for virus diagnosis. Serological reactions were evaluated by measuring optical density using a computerized spectrophotometer.

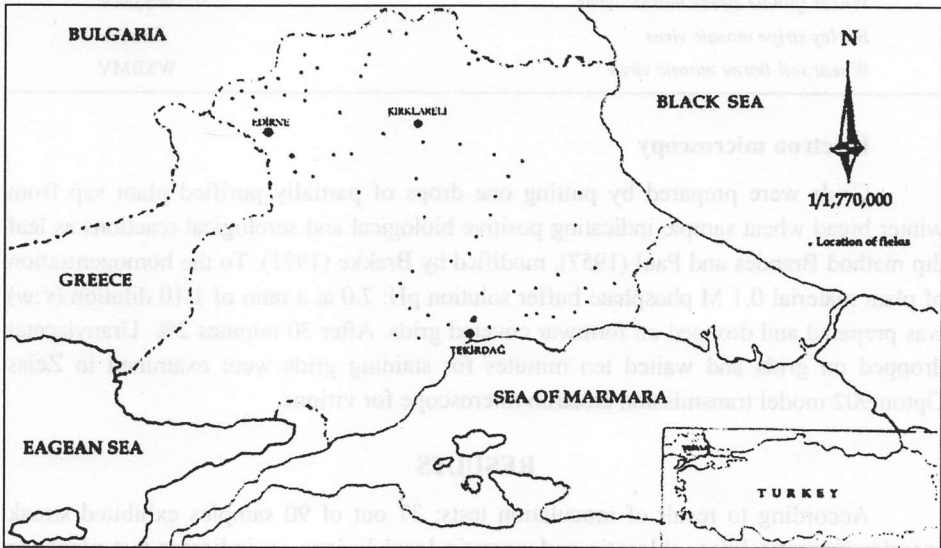


Figure 1. Map of Trakya Region of Turkey where virus diseases occurred and infected plant leaf samples were collected in 2001.

Table 1. Number of samples collected from cereal species in provinces of Trakya region

Name of species	Name of provinces			Total number of samples
	Edirne	Kırklareli	Tekirdağ	
Bread wheat	22	9	25	56
Barley	3	2	2	7
Oat	5	3	9	17
Bird seeds	-	-	8	8
Tritikale	-	-	2	2
Total number of sample in region	30	14	46	90

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Table 2. Antisera prepared against cereal viruses used for DAS-ELISA tests of infected cereal leaf samples collected in Trakya Region of Turkey.

Name of Virus Species Antisera Prepared	Abreviation
<i>Barley yellow dwarf virus -PAV</i>	BYDV-PAV
<i>Wheat dwarf virus</i>	WDV
<i>Soil-borne cereal mosaic virus</i>	SBCMV
<i>Wheat streak mosaic virus</i>	WSMV
<i>Barley yellow mosaic virus</i>	BYMV
<i>Oat necrotic mottle virus</i>	ONMV
<i>Cocksfoot mottle virus</i>	CoMV
<i>Brom sriate mosaic virus</i>	BrMV
<i>Wheat spindle streak mosaic virus</i>	WSSMV
<i>Barley stripe mosaic virus</i>	BSMV
<i>Wheat soil-borne mosaic virus</i>	WSBMV

Electron microscopy

Grids were prepared by putting one drops of partially purified plant sap from winter bread wheat sample indicating positive biological and serological reactions as leaf dip method Brandes and Paul (1957), modified by Brakke (1971). To the homogenisation of plant material 0.1 M phosphate buffer solution pH: 7.0 at a ratio of 1:10 dilution (v:w) was prepared and dropped on formwar covered grids. After 30 minutes 2% Uranylacetat dropped on grids and waited ten minutes for staining grids were examined in Zeiss Opton 902 model transmission electron microscope for virions.

RESULTS

According to result of inoculation tests; 37 out of 90 samples exhibited streak mosaic, chlorotic lines, chlorotic and necrotic local lesions on indicator test plants as listed in Table 3. Some characteristic streak symptoms are exhibited in Figure 2 on oat, wheat and barley leaves. Beside cereals *Chenopodium quinoa* L. leaves revealed chlorotic local lesions which were systemic extending to new leaves after inoculation of wheat leaf samples of No.35 and 28 wheat, barley and oat indicators inokulated with the sap of wheat samples numbered 1, 13 and 25 exhibited severe symptoms of virus symptoms. Wheat samples of No.3,4,10,19,30,31 and barley sample of No.24 gave mild symptoms after inoculated by sap. The other sampies revealed some peculiar streak symptoms on indicator barley plants.

DAS-ELISA test results revealed that nine out of 90 samples used as antigen contained *Wheat streak mosaic virus* (WSMV). Beside WSMV 63 out of 90 samples had *Barley yellow dwarf virus-PAV* (BYDV-PAV) and two out of 90 samples indicated presence *Oat necrotic mottle virus* (ONMV).

Table 3. Characteristic virus symptoms exhibited on some indicator test plants after sap inoculations made from infected cereal leaf samples

Name of provinces	Location of Samples taken	Number of samples	Cereal species	<i>Triticum aestivum</i>	<i>Hordeum vulgare</i>	<i>Avena sativa</i>	<i>Zea mays</i>	<i>Chenopodium quiona</i>
Edirne	Güllapoğlu campus	3	Wheat	Mo	Mo	Mo	-	-
	Uzgaç	13	Wheat	Mo	Mo	Mo	-	-
	Çömlekköy	15	Wheat	Mo	-	-	-	-
	Sinanköy	19	Wheat	-	Mo	Mo	-	-
	Uzgaç	25	Wheat	-	Mo	Mo	-	-
	Küküler	71	Oat	-	Mo	-	-	-
	Akardere	77	Oat	-	Mo	-	-	-
	Kırklareli yolu	83	Wheat	-	Mo	-	-	-
	Küküler	84	Oat	-	Mo	-	-	-
	Akardere	87	Oat	-	Mo	-	-	-
	Demirköy	9	Wheat	-	-	-	NS	-
Kırklareli	Malkoçlar	24	Barley	Mo	Mo	Mo	-	-
	Ata. Res. Insti.	31	Wheat	-	Mo	Mo	-	-
	Pınarhisar	69	Barley	-	Mo	-	-	-
	Türkgeldi	75	Wheat	-	Mo	-	-	-
Tekirdağ	Karadeniz M. Mev.	1	Wheat	Mo	Mo	Mo	-	-
	Lahanköy	30	Wheat	Mo	Mo	-	-	-
	Hayrabolu	4	Wheat	Mo	Mo	Mo	-	-
	Hayrabolu	10	Wheat	Mo	-	Mo	-	-
	Ferhadanlı	47	Oat	-	Mo	-	-	-
	Kayı köyü	45	Oat	-	Mo	-	-	-
	Ferhadanlı	44	Wheat	-	Mo	-	-	-
	Ferhadanlı	48	Oat	-	Mo	-	-	-
	Çukurköy	49	Oat	-	Mo	-	-	-
	Nusretfakı	51	Birdseed	-	Mo	-	-	-
	Değirmenaltı	56	Wheat	-	Mo	-	-	-
	Kayı Köyü	57	Birdseed	-	Mo	-	-	-
	Hav. Mevki	59	Wheat	-	Mo	-	-	-
	Nusretfakı	60	Barley	-	Mo	-	-	-
	Edirköy	62	Barley	-	Mo	-	-	-
	Merkez	65	Wheat	-	Mo	-	-	-
	Merkez	66	Oat	-	Mo	-	-	-
	Edirköy	86	Barley	-	Mo	-	-	-
	Sinanlı	89	Oat	-	Mo	-	-	-
Sinanlı	90	Oat	-	Mo	-	-	-	
Köscilyas	35	Wheat	-	-	-	-	CLL	
Köscilyas	35	Wheat	-	-	-	-	NLL	

Mo: Mosaic and streak mosaic, CLL: Chlorotic local lesion, NLL: Necrotic local lesion, NS: Necrotic streak

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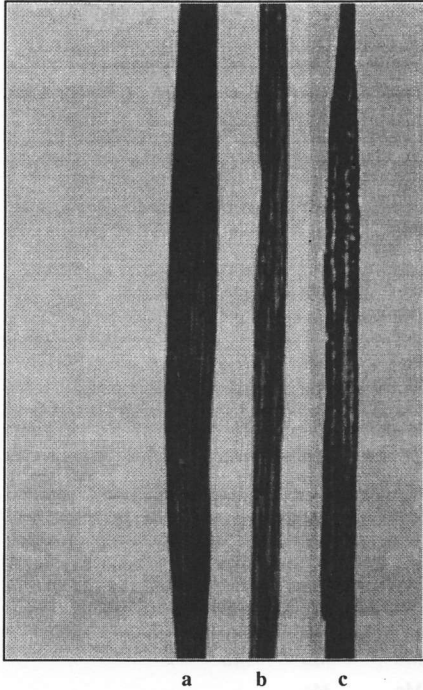


Figure 2. *Wheat streak mosaic virus* caused streak symptoms on a: oat, b: wheat and c: barley leaves.

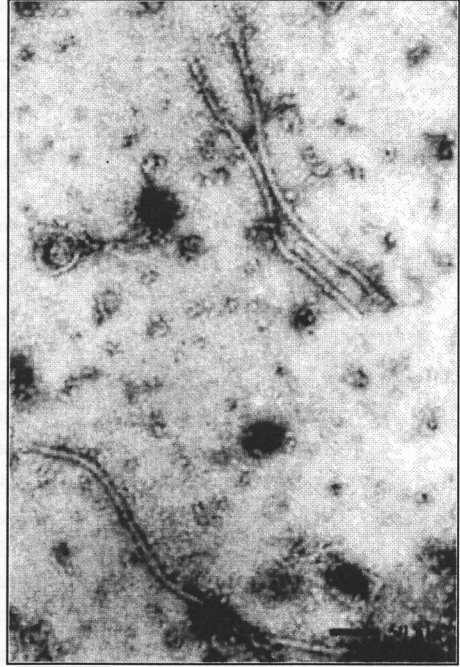


Figure 3. Electron microscopic view of *Wheat streak mosaic virus*.

Electron microscopical studies proved samples containing mechanical transmissible viruses, contained flexible rod shape virions as illustrated in Figure 3. The measured dimensions of the virion were 700- 750 nm of length and 15 nm in diameter.

DISCUSSION

Virus disease of cereals may reduce yield and quality of crop as compiled them Wiese (1996), Cook and Veseth (1991). Systemic infections on cereals revealing the yellowing, dwarfing, russeting, chlorotic streaks and yield reduction have occurred since 1999 in Trakya Region of Turkey. It might be caused by viruses. Among the viruses, a mechanically transmissible one was identified as *Wheat streak mosaic virus* (WSMV) in Trakya Region. DAS-ELISA test revealed that at least 10% of the samples contain WSMV in single or mixed with other viruses.

WSMV is a mechanically transmissible virus occurring mostly on wheat and barley. Oat is also susceptible to this virus as Brakke (1958) determined. Winter wheat *Triticum aestivum* L. is the most important host of WSMV and almost all the cultivars take place in the host range of the virus (Shahwan and Hill (1984). Our findings,

revealed in Table 3, were confirmed with the literature by indicating sap inoculation to wheat, barley and oat. As a matter of the fact, Bremer (1971) identified WSMV in the cereals in Izmir province and Ege Region in Turkey. But, her study never covered and dealt with the virus occurrence in Trakya Region.

Electron microscopy study revealed that WSMV isolate from Trakya Region contained 15x750 nm flexible rod virions as indicated in Figure 2. This dimensions of WSMV measured by some researchers whose findings were compiled by Edwardson (1974) length of virions were measured 650 nm to 830 nm. They usually measured 15x750 nm in different parts of the world. So Trakya isolate of WSMV virions take place in the same range of length as Brakke (1971) confirmed it.

By evaluating the results of mechanical inoculations, DAS-ELISA and the electronmicroscopy measurements of virions convinced of the presence of WSMV and it could be considered as one of the major virus pathogens infecting wheat and barley in Trakya Region of Turkey.

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ÖZET

TÜRKİYE'NİN TRAKYA BÖLGESİNDE TAHILLARDA ENFEKSİYONLARA NEDEN OLAN BİR VİRUS *Wheat Streak Mosaic Virus* (WSMV)

Türkiye'nin Trakya Bölgesi'nde 1999 yılından beri kışlık ekmeklik buğday başta olmak üzere tahıllarda sistemik enfeksiyonlara neden olan virüslerden birisi *Wheat streak mosaic virus* (WSMV) olarak tanılanmıştır. Bitkilerde şiddetli mozaik, kardeşlenen sürgünlerde nekrozlar, cücelik ve özellikle kışlık ekmeklik buğdayda verim düşüklüğü karakteristik belirtiler olarak gözlenmiştir. Bölgede'ki 54 farklı tarladan belirtiler gösteren bitkilerden oluşan 90 ayrı örnek toplanarak bunlardan elde edilen öz suları sekiz tür bitkiden oluşan indikatör bitki serilerine mekanik olarak inokule edilmişlerdir. 10 yaprak örneğinin içerdiği virus iki ayrı indikatör bitki türünde virus belirtilerine neden olurken 27 örneğin öz sularının aşılandığı serilerden en az bir türün virüsle bulaştığı saptanmıştır.

Yine 11 farklı tahıl virüsüne karşı hazırlanmış antiserumlar kullanılarak örneklere Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) testleri uygulanmıştır. Sonuçta 90 adet örnekten 9 tanesinde WSMV'nun varlığı serolojik olarak kanıtlanmıştır. WSMV içeren kışlık ekmeklik buğday örneğinden Transmission elektron mikroskop (TEM) için hazırlanan gridlerin esnek çubuk formunda 15x750 nm boyutlarında virionlar içerdikleri görülmüştür. Elde edilen biyolojik, serolojik ve

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elektron mikroskopik bulgular Trakya Bölgesi'nin tahıl tarlalarında WSMV'den kaynaklanan enfeksiyonlar olduğunu göstermiştir.

Anahtar Kelimeler: Buğday çizgi mozaik virüsü, ELISA, tahıl, virüs

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The Use of RT-PCR for Specific Detection of *Apple mosaic virus* (ApMV) in Apple

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ABSTRACT

RT-PCR (reverse transcription-polymerase chain reaction) assays using DNA primers for the viral coat protein region were employed to test *Apple mosaic virus* (ApMV) in apple trees. Total RNA was extracted from apple leaves showing ApMV symptoms from young shoots by using lithium chloride based buffer. The expected size of PCR amplified products was 680 bp. The detection of ApMV by RT-PCR was correlated with ELISA assays. Primers were highly specific for the detection of ApMV and they did not react with any of the other examined viruses including *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple chlorotic leaf spot virus* (ACLSV). RT-PCR assays were successful in the detection of several isolates of ApMV and appeared useful for testing apple germplasm in quarantine or budwood certification programme.

Key words: ApMV, apple, RT-PCR

INTRODUCTION

Apple mosaic virus (ApMV) is a member of *Ilarvirus* genus of *Bromoviridae* family and occurs world-wide wherever apple and the other hosts are grown. Virus has a wide host range such as apple (*Malus* spp.), apricot, cherry, almond, plum and peach (*Prunus* spp.), blackberry, raspberry (*Rubus* spp.), hazelnut (*Corylus avellana*), roses (*Rosa* sp.) and hop (*Humulus lupulus*) (Brunt et al., 1996). No insect vector is known and the virus is transmitted by pollen, by vegetative propagation from infected trees or by mechanical inoculation (Nemeth, 1986).

Although the symptoms of ApMV show considerable variation according to cultivar susceptibilities and virus strains, virulent strains causes pale yellow banding or cream coloured mottling, consisting of smaller and larger spots, or ringspots (Nemeth, 1986). The economic damage of ApMV was determined as reducing growth by 50%, trunk diameter by 20% and crop yield by 30% (Nemeth, 1986).

THE USE OF RT-PCR FOR SPECIFIC DETECTION OF *APPLE MOSAIC VIRUS* (ApMV) IN APPLE

Reliable detection of ApMV is required in certification schemes in order to provide virus-free propagation material for fruit tree growing areas and to prevent entry of imported ApMV infected plant material. Although a bioassay, which is based on grafting buds or chips onto an indicator plants, has been used extensively for reliable detection of APMV, it is time consuming, season dependent and cannot be completed in areas where the proper indicator species will not grow. Testing for ApMV by ELISA (Enzyme-linked immunosorbent assay) has superceeded the bioassay to some extent. Its routine use is hampered by some factors such as unevenly distribution of the virus throughout the buds and limbs of infected trees, fluctuation of virus concentration during individual growing seasons and between seasons, and no reaction of all ApMV isolates to a specific antiserum (Sanchez-Navarro et al., 1998, Dal Zotto et al., 1999).

Most of the plant viruses especially in problematic plant material e.g. ligneous tissue of fruit trees (Korschineck et al., 1991; Rowhani et al., 1995), gladiolus corms (Vunsh et al., 1991), potato tubers and micro tubers (Spiegel and Martin, 1993) have been detected using RT-PCR method since the application of PCR techniques to plant virology (Saiki et al., 1985). RT-PCR allows detection of low concentration of virus, undetected by ELISA, in leaf and bark samples collected from dormant trees during winter (Spiegel et al., 1996).

The use of RT-PCR technique, which is the most sensitive but also the less affordable and time consuming (Sanchez-Navarro et al., 1998), might be useful in overcoming some of obstacles in virus indexing for sanitation purposes. In this report we demonstrate the adoption and usefulness of RT-PCR assays to test for ApMV Turkish apple isolates.

MATERIALS and METHODS

Plant material: ApMV infected apple plant materials were provided from National Fruit Nursery Station, Tokat. Three apple samples belong to Granny Smith apple cultivars showing typical ApMV symptoms were used and thirteen apple samples from Ankara were tested.

ELISA: DAS-ELISA (Double antibody sandwich-enzyme linked immunosorbent assay) using ApMV-specific polyclonal antiserum (Loewe, Sauerlach/Germany) was performed according to Clark and Adams (1977).

RNA extraction: For RNA extraction of total RNA's, a lithium chloride-based method was used according to Spiegel et al (1996). Each sample (100 mg of fresh or 25 mg of CaCl₂ dried leaf pieces exhibiting virus symptom) was homogenised using pestle and mortar with 5 volumes of extraction buffer (500 µl; 200 mM Tris-HCl pH 8.5, 1.5% sodium dodecylsulphate, 300 mM lithium chloride, 10 mM EDTA, 1% sodium deoxycholate, 1% Igepal) and 0.5% 2-mercaptoethanol. The extract was collected in a 1.5 ml microfuge

tube and heated for 15 minutes to 65°C. Then equal volumes of 6 M potassium acetate pH 6.5 were added. After mixing by vortex, the tubes were incubated in ice for 15 minutes and then centrifuged at 12 000 g for 15 minutes. Nucleic acids were precipitated from the supernatant with isopropanol and collected by centrifugation as described above. The pellet was resuspended after drying in 50 µl sterile water.

Primers and RT-PCR amplification protocol: An artificial RT-PCR primer set designed for detection of ApMV in apple (Lee et al., 1998) was used. Sense primer was 5'-TCA ACA TGG TCT GCA AGT AC-3' and the antisense primer was 5'-CTA ATC GCT CCA TCA TAA TT-3' amplifying 680 bp long fragment derived from the CP gene on RNA3.

The reverse transcription (RT) and PCR were performed following a single non-interrupted thermal cycling program (Spiegel et al., 1996). The total volume of reaction was 25 ml and each reaction contained the RNA template (about 1 µg), virus specific primers (0.8 µM each), 400 µM dNTPs, 1.5 mM MgCl₂, 2.5 µl 10x reaction buffer (final concentration of 10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.08% Igepal), 1 unit of Taq DNA polymerase (Promega), 0.8 unit of M-MLV reverse transcriptase (MBI Fermentas) and 0.8 unit RNase inhibitor (MBI Fermentas). The amplification protocol was as follows: 60 min at 37°C for reverse transcription, one cycle of 4 min at 94°C, 2 min at 54°C, 3 min at 72°C; one cycle of 2 min at 94°C, 2 min at 54°C, 3 min at 72°C, than 40 cycles 30 sec at 94°C, 1 min at 54°C, 1 min at 72°C and finally 10 min at 72°C. PCR products were analysed by electrophoresis in a 1% agarose gel, stained with ethidium bromide (EtBr) and viewed under UV.

RESULTS and DISCUSSION

ApMV symptoms that are pale yellow banding or cream coloured mottling, consisting of smaller and larger spots, or ringspots were observed on the samples obtained from Tokat National Fruit Nursery Station. DAS-ELISA against ApMV gave A405 0.648 which was almost thrice higher than the value of negative control. Thus, DAS-ELISA confirmed that the symptoms observed on the apple leaves were specific to ApMV.

The LiCl method used for nucleic acid isolation for RT-PCR was simple and there was no need for extraction with phenol/chloroform. RNA yield was 4.3 µg total RNA per 100 mg of fresh leaf tissue.

In order to study the possibility of infection with additional common viruses of pome fruits, plant samples were also tested for some other viruses by RT-PCR. Results of the amplifications using PNRSV (*Prunus necrotic ringspot virus*), PDV (*Prune dwarf virus*) and ACLSV (*Apple chlorotic leaf spot virus*) specific primers yielded no amplified bands (Figure 1a).

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For optimising RT-PCR system for ApMV, optimum ApMV specific primer concentrations were determined: 50, 25, 12, 6 and 3 pmol/ μ l of primer concentrations were prepared and RT-PCR was performed. A specific DNA band of about 680 bp was visible in each primer concentration in EtBr stained gel (Figure 1b). The decrease in primer concentration resulted in weaker bands of amplified products. Thus, 20-25 pmol/ μ l of primer concentrations were selected for further ApMV RT-PCR assays. Primer concentrations higher than 50 pmol/ μ l inhibited the amplification and no band was observed in the gel (data not shown).

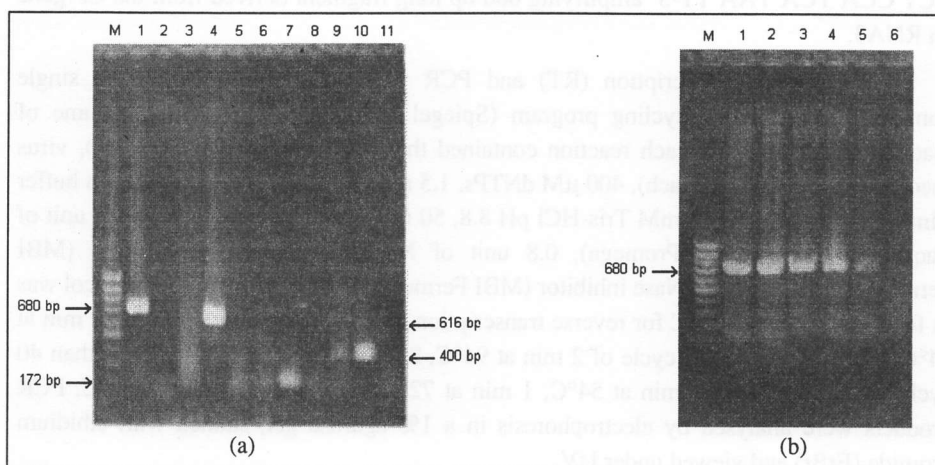


Figure 1. a) RT-PCR test of RNA extracted from apple against to ApMV, PNRSV, PDV and ACLSV; 1,2 ApMV primers; 3, 4, 5 PNRSV primers; 5, 6, 7 PDV primers; 7, 8, 9 ACLSV primers were used; 2, 5, 8, 11 without RNA; 4, 7, 10 plasmid clones of PNRSV, PDV and ApMV instead of RNA as control, respectively.

b) RT-PCR in different concentration of ApMV specific primer pair. 1, 2, 3, 4, 5, 6 correspond to 50, 25, 12, 6, 3 pmol/ μ l respectively; M Marker 1000 bp DNA ladder.

Several viruses are commonly widespread in Turkey including: PNRSV, PDV and ACLSV. Possibility of cross reaction of ApMV primer pair against to those viruses by RT-PCR was demonstrated on comparisons with primer pairs unique to each of these viruses. RT-PCR was carried out using ApMV specific primers with RNAs extracted from infected plant samples by PNRSV, PDV and ACLSV isolates obtained from different part of Turkey. ApMV specific primers did not yield any PCR products cross-reacted with RNA of the other viruses infecting apple (Figure 2). Thus, the primer set used for detection of ApMV was convenient and it produced a 680 nt fragment from the ApMV RNA3 template. On the other hand, thirteen apple plant samples provided from Ankara were tested by RT-PCR system and 3 of them showing APMV mosaic symptoms were found infected by the virus.

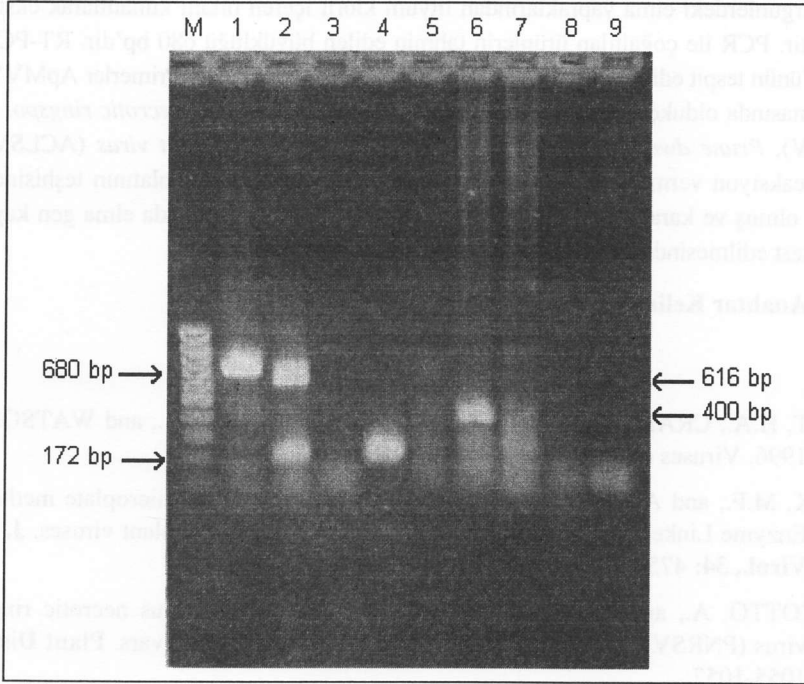


Figure 2. RT-PCR test of RNA extracted from ApMV (1), PNRSV (2, 3), PDV (4, 5) and ACLSV (6, 7) infected plant by using ApMV specific primer pair. M 1000 bp DNA ladder; 2, 4, 6 correspond to PCR products amplified with specific primers of PNRSV, PDV and ACLSV as control, respectively. 8 represents healthy plant RNA, 9 without RNA.

This report demonstrates the specificity of ApMV detection of RT-PCR in Turkish apple isolates. This technique might be used in apple germplasm collections alternatively to serological methods and bioassays in order to virus indexing for sanitation purposes.

ACKNOWLEDGEMENTS

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ÖZET

ELMADA APPLE MOSAIC VIRUS (ApMV)'ÜN SPESİFİK TANISINDA RT-PCR'İN KULLANIMI

RT-PCR (revers transkripsiyon-polimeraz zincir reaksiyonu) yöntemi viral kılıf protein bölgesindeki DNA primerleri kullanılarak elma ağaçlarındaki *Apple mosaic virus* (ApMV)'ün test edilmesinde kullanılmıştır. Toplam RNA ApMV belirtileri gösteren

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genç sürgünlerdeki elma yapraklarından lityum klorit içeren ortam kullanılarak ekstrakte edilmiştir. PCR ile çoğaltılan ürünlerin tahmin edilen büyüklüğü 680 bp'dir. RT-PCR ile ApMV'ünün tespit edilmesi ELISA testlerine uygunluk göstermiştir. Primerler ApMV'ünün tanımlanmasında oldukça spesifik olmuş ve araştırılan diğer *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) ve *Apple chlorotic leaf spot virus* (ACLSV) ile hiçbir reaksiyon vermemiştir. RT-PCR testi diğer birkaç ApMV izolatının teşhisinde de başarılı olmuş ve karantina ve aşı kalemi sertifikasyon programlarında elma gen kaynaklarının test edilmesinde kullanışlı olduğu ortaya konmuştur.

Anahtar Kelimeler: ApMV, elma, RT-PCR

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Determination of Some Viruses Infecting Common Bean (*Phaseolus vulgaris* L.) and Their Incidences in Seed Lots in Samsun Province

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ABSTRACT

DAS-ELISA tests performed using 499 symptomatic plants, which were collected from common bean fields in Samsun province in 2002, showed that 36% of the samples were infected with *Bean common mosaic virus* (BCMV), 10.8% with *Cucumber mosaic virus* (CMV), 2.8% with *Bean common necrosis virus* (BCMNV) and 2% with *Bean yellow mosaic virus* (BYMV). Some plants had mixed virus infections while most of them (84.6%) were singly infected. The most common mixed infection was BCMV+CMV (17 samples; 3.41%) double infection. The only two plants (0.4%) were infected with all viruses tested.

In this study, 53 seed samples were collected from local growers (42 samples) and commercial seed stores (11 samples), and primary leaves of each seed sample were tested by DAS-ELISA. Of the seeds tested, 18.9% were infected with BCMV, 17% with BCMNV and 17% with CMV. None of the seed samples were found to be infected with BYMV. Out of 29 seed samples tested, 4 (13.7%) were found to be positive for *Alfalfa mosaic virus*. This is the first study which distinguishes BCMNV infection from BCMV infection in common bean growing areas in Turkey. Necessity of limiting BCMNV spread by sowing the seeds of varieties carrying resistance gene is discussed.

Key words: *Phaseolus vulgaris*, bean, virus, seed transmission

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is one of the most important crops in Samsun province with a total of 81.435 ton fresh bean and 14.268 ton dry bean production in a year. This is about 16.9% of fresh bean and 6% of dry bean production of Turkey (Anonymous, 2001).

Thirty four viruses are reported to naturally infect the common bean worldwide (Morales and Bos, 1988). So far, *Bean common mosaic virus* (Açikgöz, 1984; Fidan and Yorgancı, 1990; Lisa et al., 1994; Gümüş et al., 2001; Kutluk Yılmaz et al., 2002), *Bean yellow mosaic virus* (Açikgöz, 1984; Lisa et al., 1994), *Cucumber mosaic virus* (Lisa et al.,

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1994), *Tobacco black ring virus* and *Alfalfa mosaic virus* (Gümüş et al., 2001) infections were detected in common bean in Turkey.

In the field, typical virus symptoms observed in Samsun province were green mosaic, green vein banding, mottling, malformation, upward curling of leaves and yellow mosaic. The main aim of this study was to determine bean-infecting viruses in Samsun by a serological survey. The presence of these viruses in seed samples collected from local farmers and commercial seed stores in the province were also determined.

MATERIALS and METHODS

Sample Collection

Sampling studies were performed in 65 bean fields which were randomly selected in the most bean growing districts (Bafra, Çarşamba, Tekkeköy and Terme) in 2002. Two or three apical leaf samples were taken from symptomatic plants. Samples were collected in an X-shaped pattern across the field. All samples were put into polythene bags and stored at -20°C until used.

Serological Assay on Leaf Samples

Double antibody sandwich-enzyme linked immunorbent assay (DAS-ELISA) method was applied according to Clark & Adams (1977) and instructions of the antisera's manufacturer (Loewe Biochemica, Germany). Leaf samples were ground (1g leaf/10 ml buffer) in extraction buffer (PBS: 0.13 M NaCl, 0.014 M KH₂PO₄, 0.08 M Na₂HPO₄·12H₂O, 0.002 M KCl, pH: 7.4) containing 0.05% Tween-20, 0.1% skimmed milk powder and 2% polyvinyl pyrrolidone (PVP-40). The extracts were added to wells of microplates (TPP, Switzerland) coated with BCMV, BYMV, *Bean common mosaic necrosis virus* (BCMNV) and CMV-specific polyclonal antisera diluted in carbonate buffer (pH: 9.6) and incubated at 4°C overnight. Plates were washed three times with PBST/Tween-20 buffer between each step, and alkaline phosphatase conjugated antibody diluted in extraction buffer was added. After washing, p-nitrophenyl phosphate in diethanolamine substrate buffer (0.5 mg/ml, pH: 9.8) was added to the wells and incubated at room temperature for 30-180 min. Absorbance values were read at 405 nm using a microplate reader (Tecan Spectra II, Austria). A sample was considered to be positive if the A405 value was two fold greater than the mean of healthy bean samples (Davis and Hampton, 1986).

Serological Assay on Seed Samples

Fifty-three seed samples of the common bean collected from growers and commercial producer were screened for infection by BCMV, BCMNV, BYMV and CMV. Only 29 samples were tested for *Alfalfa mosaic virus* (AMV) infection due to insufficient antiserum to complete testing all seed samples. Seeds were previously germinated in the Petri dishes

containing double layer of wetted filter paper in a group of 50 seeds for each sample, and primary leaves of each seedling were mixed together and tested by DAS-ELISA.

RESULTS and DISCUSSION

A total of 499 leaf samples were collected during random surveys in a total of 65 bean fields in Bafra, Çarşamba, Tekkeköy and Terme districts in 2002 growing season. BCMV, CMV, BCMNV and BYMV were identified in 36% (180 samples), 10.8% (54 samples), 2.8% (14 samples) and 2% (10 samples), respectively (Table 1), of the samples taken from plants expressing virus-like symptoms.

The only 43% (215 samples) of the plants were found to be positive with any of the four viruses tested. The result indicated that some samples were possibly infected with other bean-infecting viruses, but they were not detected because their antisera were not available in the current research. BCMV and CMV were the most common viruses in bean fields in Samsun. Similarly, Lisa et al. (1994) found that BCMV (27.8%) and CMV (5.6%) were the most detected viruses in bean samples collected in Turkey. In the current study, BYMV infection was found to be relatively low (2%), possibly because of the fact that it is not seed-transmitted in common bean. However, it is transmitted by the seeds of some other legumes such as *Medicago polymorpha* (0.9%), *Medicago truncatula* (0.3%), *M. indica* (1%), *Trifolium arvense* (0.1%), *T. campestre* (0.2%) and *T. glomeratum* (0.05) (McKirby and Jones, 1995).

The percentage of single infections was 84.6% (182 samples). However, some samples had mixed virus infections and the most common mixed infection was BCMV+CMV (3.4%; 17 samples) double infection. Other double infections determined were BCMV+BCMNV (1.2%; 6 samples) and BCMV+BYMV (0.4%; 2 samples). The percentages of triple infections were 0.6% (3 samples), 0.4% (2 samples) and 0.2% (1 sample) for BCMV+BCMNV+CMV, BCMV+BCMNV+BYMV and BCMV+CMV+BYMV, respectively (Figure 1). The result of DAS-ELISA showed that 0.4% of the samples (2 samples) were mixed infected with BCMV, BYMV, BCMNV and CMV (Figure 1).

Viruses sometimes could be more destructive if two or more viruses simultaneously infect the same plant. BCMV+BYMV mixed infections caused high yield reductions due to synergistic effect of these viruses on bean (Stanyulis, 1976). In the present work, synergistic or antagonistic effects of mixed virus infections on bean were not studied. However, further study is needed to reveal if any synergism or antagonism occurs between viruses.

This is the first study which differentiates BCMNV and BCMV infections in bean growing areas of Turkey. BCMV and BCMNV were initially considered to be one virus. Later, they were separated according to host reactions, serological assays and genomic sequences (Coyne et al., 2003). It was first proposed that they were two

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Table 1. Occurrence of viruses in leaf samples collected during surveys from 65 fields in 28 villages in Samsun province in 2002.

Districts	Villages	No. of fields surveyed	No. of samples tested	BCMV	BYMV	CMV	BCMNV
BAFRA	Sarıkaya	3	15	12	0	0	0
	Sarıköy	3	15	9	1	0	0
	Adaköy	2	8	6	0	0	0
	Total	8	38	27 (71%)	1 (2.6%)	0 (0%)	0 (0%)
ÇARŞAMBA	Karamustafalı	5	43	14	1	1	1
	Kaydan	2	12	2	0	2	0
	Y. Dikencik	4	43	3	0	2	1
	Y. Kavacık	2	12	5	0	0	0
	Ahubaba	2	16	10	1	1	1
	Ovacık	6	39	23	1	3	2
	Yeniköseli	3	22	4	2	7	4
	Damlataş	3	22	7	0	2	1
	Bafracalı	4	35	2	1	0	0
	Merkez	1	10	5	2	2	0
	Beylerce	2	11	2	0	0	0
	Alibeyli	2	13	3	0	0	0
	Yamanlı	2	13	4	0	0	0
	Durusu	1	2	0	0	0	0
Elifli	1	3	1	0	0	0	
Total	40	296	85 (28.7%)	8 (2.7%)	20 (6.7%)	10 (3.3%)	
TEKKEKÖY	Merkez	1	14	9	0	9	1
	Kutlukent	2	16	7	0	8	1
	Selyeri	1	10	2	0	8	0
	Ovabaşı	2	15	2	0	1	0
	Total	6	55	20 (36.3%)	0 (0%)	26 (47.2%)	2 (3.6%)
TERME	Uzungazi	2	15	1	0	4	0
	Bağsaray	2	30	7	0	0	0
	Merkez	2	15	3	0	0	0
	Kozluk	2	30	25	1	0	2
	Geçmiş	1	8	5	0	0	0
	Sarayköy	2	12	7	0	4	0
	Total	11	110	48 (43.6%)	1 (0.9%)	8 (7.2%)	2 (1.8%)
TOTAL	65	499	180 (36%)	10 (2%)	54 (10.8%)	14 (2.8%)	

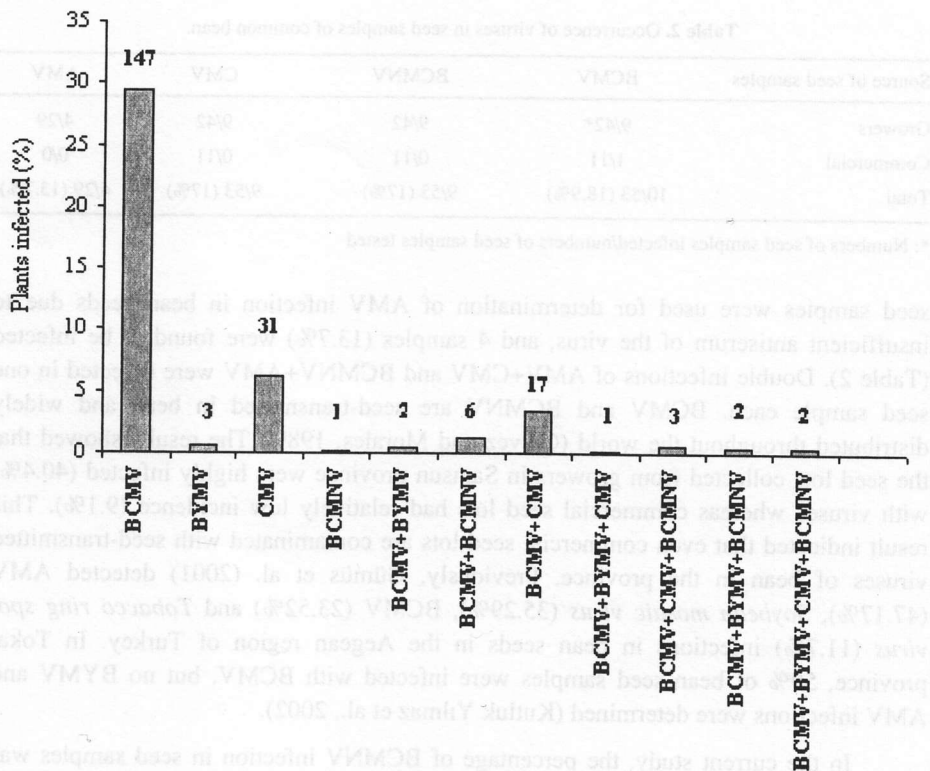


Figure 1. The percentages of single and mixed infections of viruses. The numbers above boxes indicate the numbers of plants infected.

different strains (Drijfhout et al., 1978) and then two distinct viruses (McKern et al., 1992, Vetten, et al., 1992; Mink et al., 1994). Both BCMV and BCMNV cause similar symptoms on varieties without I gene, but the only BCMNV gives systemic necrosis (called black root) in bean varieties containing the I gene at growing temperatures (less than 30°C). These varieties carrying the gene I confer high levels of resistance to all BCMV isolates at growing temperatures (Gilbertson et al., 2001).

In this study, 53 seed samples were collected from growers and commercial seed lots in Samsun province. The primary leaves of seedlings belonging to each seed sample were mixed together and tested by DAS-ELISA. Of the seeds tested, 18.9% (10 samples) were infected with BCMV, 17% (9 samples) with BCMNV and 17% (9 samples) with CMV (Table 2). BCMNV was detected in mixed infections in common bean seed samples. Triple infection of BCMV, BCMNV and CMV were determined in four samples. Four seeds were also double-infected with BCMV and BCMNV. None of the seed samples were found to be infected with BYMV (data not shown). Only twenty nine

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Table 2. Occurrence of viruses in seed samples of common bean.

Source of seed samples	BCMV	BCMNV	CMV	AMV
Growers	9/42*	9/42	9/42	4/29
Commercial	1/11	0/11	0/11	0/0
Total	10/53 (18.9%)	9/53 (17%)	9/53 (17%)	4/29 (13.7%)

*: Numbers of seed samples infected/numbers of seed samples tested

seed samples were used for determination of AMV infection in bean seeds due to insufficient antiserum of the virus, and 4 samples (13.7%) were found to be infected (Table 2). Double infections of AMV+CMV and BCMNV+AMV were detected in one seed sample each. BCMV and BCMNV are seed-transmitted in bean and widely distributed throughout the world (Galvez and Morales, 1989). The results showed that the seed lots collected from growers in Samsun province were highly infected (40.4%) with viruses whereas commercial seed lots had relatively low incidence (9.1%). This result indicated that even commercial seed lots are contaminated with seed-transmitted viruses of bean in the province. Previously, Gümüs et al. (2001) detected AMV (47.17%), *Soybean mosaic virus* (35.29%), BCMV (23.52%) and *Tobacco ring spot virus* (11.7%) infections in bean seeds in the Aegean region of Turkey. In Tokat province, 59% of bean seed samples were infected with BCMV, but no BYMV and AMV infections were determined (Kutluk Yılmaz et al., 2002).

In the current study, the percentage of BCMNV infection in seed samples was 17%. These results suggested that common bean varieties sown in the region may not contain the I gene for BCMNV resistance. Because BCMNV cause systemic necrosis reaction and cannot be carried in the seeds of varieties that possess the I gene. The spread of BCMNV was limited by early identification of the virus and not taking seeds from BCMNV-infected plants for production in California (Gilbertson et al., 2001). The incidence of BCMNV infection in field samples was relatively low (2.8%). It may be possible to minimize the spreading of BCMNV in bean growing areas of Turkey if the seeds of varieties with I gene are used for planting. Knowing the virus and its strains are also important for reliable breeding for resistance to bean viruses. Therefore, there is a need to investigate BCMV and BCMNV strains and reactions of bean varieties to them in Samsun and other parts of Turkey.

ÖZET

SAMSUN İLİNDE FASULYE (*Phaseolus vulgaris* L.)'DE ENFEKSİYON OLUŞTURAN BAZI VİRÜSLERİN VE TOHUMDA BULUNUŞ ORANLARININ BELİRLENMESİ

Samsun'da fasulye yetiştirilen alanlardan 2002 yılında toplanan 499 simptomlu yaprak örneğinin, DAS-ELISA yöntemi ile test edilmesi sonucunda, %36'sının *Bean*

common mosaic virus (BCMV), %10.8'inin *Cucumber mosaic virus* (CMV), %2.8'inin *Bean common mosaic necrosis virus* (BCMNV) ve %2'sinin *Bean yellow mosaic virus* (BYMV) ile enfekteli olduğu belirlenmiştir. Pozitif örneklerin büyük bir kısmının (%84.6; 182 örnek) test edilen 4 virüsten herhangi birisi ile enfekteli olduğu belirlenmiştir. Birden fazla virüs ile enfekteli örnekler arasında en fazla BCMV+CMV ikili enfeksiyonuna (%3.4) rastlanmıştır. Sadece 2 bitkinin (%0.4) BCMV, BCMNV, BYMV ve CMV ile karışık olarak enfekteli olduğu saptanmıştır.

Bu çalışmada, 42 üretici ve 11 ticari olmak üzere 53 fasulye tohum örneği toplanmıştır. Her örneğe ait 50'şer tohumun Petri kabında çimlendirme sonrası oluşan primer yaprakları, toplu halde DAS-ELISA yöntemi ile test edilmiştir. Tohumların %18.9'unun BCMV, %17'sinin BCMNV ve %17'sinin CMV ile bulaşık olduğu belirlenmiştir. BYMV ile bulaşık tohum örneğine rastlanılmamıştır. Ayrıca, test edilen 29 tohum örneğinin 4 (%13.7)'ü AMV ile bulaşık olarak belirlenmiştir. Bu çalışma, Türkiye'de fasulyede BCMV ve BCMNV enfeksiyonunun birbirinden ayrılmasını sağlayan ilk araştırmadır. BCMNV'nin daha fazla yaygın hale gelmemesi için virüse dayanıklı çeşitlerin belirlenmesi ve bu çeşitlerin tohumlarının üretimde kullanılmasının gerekliliği vurgulanmıştır.

Anahtar Kelimeler: *Phaseolus vulgaris*, fasulye, virus, tohumla taşınma

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Detection of *Plum pox virus* (PPV) Prevalence in Stone Fruit Trees of Turkey by RT-PCR

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ABSTRACT

Plum pox virus (PPV) infection on stone fruit trees was investigated in Afyon, Amasya, Burdur, Bursa, Çanakkale, Isparta, Izmir and Yalova provinces by using molecular techniques based on polymerase chain reaction (PCR). A total of 361 stone fruit tree samples including sweet- and sour- cherry, peach, nectarine, apricot and plum were tested by RT-PCR (Reverse Transcription-PCR) and 12 of them were detected as PPV infected. The infections were recorded on plum, apricot, peach and nectarine trees but not in sour- and sweet cherry trees. In the present study, the tested stone fruit trees from central and western part of Turkey, except from Yalova and Bursa, were found to be free of PPV and no infection was detected on sour- and sweet-cherry trees.

Key words: *Plum pox virus*, RT-PCR, IC-RT-PCR, Turkey

INTRODUCTION

Plum pox virus (PPV), a member of the *Potyvirus* genus (Family Potyviridae) is the causal agent of the Sharka disease, which is thought to be one of the most important diseases of stone fruit trees, because of its large agronomic impact and economic importance (Sutic et al., 1999). Although not a danger to consumers, PPV can ruin the fruit's marketability by increasing acidity and causing deformities. PPV has its origin in Eastern Europe (Bulgaria) and has spread from there to most of the continent by aphid vectors and infected plant material. Except Europe, the virus is present in Asia including Azerbaijan (unconfirmed), Cyprus, Georgia (unconfirmed), India, Syria and Turkey; in Egypt, Chile (eradicated) and New Zealand (unconfirmed) (EPPO, 2004). Among its hosts, there are a number of economically important stone fruit species such as plum, apricot and peach (Nemeth, 1986). PPV was also reported recently on sour cherry (Kalashyan and Bilkey, 1989; Kalashyan et al., 1994), sweet cherry (Crescenzi et al. 1997), almond (Pribek and Gaborjanyi, 1997) and walnut (Baumgartnerova, 1996).

PPV symptoms may appear on leaves as chlorotic spots, bands or rings, vein clearing, or even leaf deformation in peaches; infected fruits show chlorotic spots or rings on the surface, and diseased plums and apricots are deformed and show internal browning of the flesh, their stones show pale ring or spots (Nemeth, 1986; Sutic et al.,

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1999). Currently, four subgroups of PPV isolates are recognised on the basis of their serological properties and molecular variability. These are two major clusters of PPV isolates representing Dideron (D) and Marcus (M) serotypes of the virus (Kerlan and Dunez, 1979), the divergent Egyptian strain PPV-El Amar (Wetzel et al., 1991) and the recently described unique strain PPV-C (cherry) which naturally infects sweet and sour cherry (Kalashyan and Bilkey, 1989; Nemchinov and Hadidi, 1996; Crescenzi et al., 1997). PPV-M is readily transmitted by aphids to peach, plum, apricot, whereas PPV-D is little or not all aphid transmissible to and between peaches (Quiot et al., 1995). Thus, M serotype is the most aggressive isolate.

The presence of PPV in Turkey was reported from Marmara and Central Anatolia Regions (Şahtiyancı, 1968; Kurçman, 1973). The Eastern Mediterranean Region and East Anatolia were reported as free of sharka (Çağlayan and Gazel, 1998; Sipahioğlu, 1999; Yorgancı et al., 2001). Turkish PPV isolates were considered to be M serotype based on PCR, but possessing both D- and M- specific epitopes (Boscia et al., 1997; Myrta et al., 1998; Candresse et al., 1998; Sertkaya et al., 2003).

Since PPV is considered to be a quarantine pathogen in many countries, substantial efforts are being made to survey and control the spread of the disease. In order to achieve this goal is needed to survey and determine the incidence of the virus in a country. By the point of view, the aim of this investigation was to search PPV incidence in central and west part provinces of Turkey, in which no detailed survey has been performed, by using RT-PCR which is sensitive and rapid tool for detecting PPV, and to adopt immuno-capture RT-PCR system to Turkish isolates.

MATERIALS and METHODS

Plant material: Samples were collected from the provinces, in which stone fruits are grown extensively, including Afyon, Amasya, Burdur, Bursa, Çanakkale, Isparta, İzmir and Yalova in May-June, 2000-2001 (Table 1). Sour and sweet cherry, peach, apricot, nectarine and plum trees were sampled both in nurseries and orchards. Shoots were selected from trees showing the disease symptoms or randomly, and placed in plastic sample bag labelled with the location. PPV infected apricot from Ankara was used as positive control and uninfected GF-305 peach leaves was included as negative control in the experiments.

RNA isolation: A lithium chloride-based method (Spiegel et al., 1996) with slight modifications as described in Ulubaş et al. (2004) was used for the isolation of total RNA's.

Immuno capture (IC): Loewe (Germany) and Bioreba (Switzerland) antisera were used for immuno-capturing of the viral RNA. Two protocols with modifications were

Table 1. Amount of stone fruit tree samples collected from different provinces in Turkey in 2000-2001.

Stone Fruit Species	Sample Collection Provinces								
	Afyon	Amasya	Burdur	Bursa	Çanakkale	Isparta	Izmir	Yalova	TOTAL
Sweet cherry	49	47	1	1	6	2	76	2	184
Sour cherry	1	-	-	-	-	2	5	8	16
Peach	-	12	16	13	5	4	11	6	67
Nectarin	-	-	7	7	9	-	-	4	27
Apricot	6	3	-	-	-	-	1	5	15
Plum	2	5	1	1	1	8	17	17	52
TOTAL	58	67	25	22	21	16	110	42	361

followed for coating and capturing the virus from the plant extracts. The first protocol (Wetzel et al., 1992) was to coat thin walled PCR tubes by antisera (100 µl) diluted (1:200 Loewe, 1:1000 Bioreba) in carbonate buffer (pH 9.6) at 37°C for 4 hours and then to wash by PBS-tween. Fresh leaves (100 µg) were ground in 1 ml PBS containing 2% PVP-40, after quick spin, they were added (100 ml) to precoated tubes, then maintained at room temperature for over night. After washing, the tubes were used immediately by filling with RT-PCR mixture (without RNA) or stored at -20°C. The second protocol (Rosner et al., 1998) was followed by the same procedure but the antisera were diluted in 0.1 M borate buffer (pH 8.5) and plant materials were extracted in TBS (10 mM Tris-HCl pH 7.4, 8 g/l NaCl and 20 g/l PVP-40) buffer. The tubes were washed with TBS-tween solution.

Primers: The DNA primers were specific for 3' non-coding region (NCR) of PPV RNA. Universal NCR-3' end primer was 5'- GTA GTG GTC TCG GTA TCT ATC ATA - 3', and universal NCR-5' end primer was 5'- GTC TCT TGC ACA AGA ACT ATA ACC - 3' (Levy and Hadidi, 1994). The amplified fragment size was 220 base pairs (bp).

RT-PCR: The total volume of reaction was 25 µl for RT-PCR (50 µl for IC-RT-PCR) and each reaction contained the RNA template (about 1 µg; without extracted RNA for IC-RT-PCR), virus specific primers (0.8 µM each), 400 µM dNTPs, 1.5 mM MgCl₂, 2.5 µl 10x reaction buffer (final concentration of 10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.08% Igepal), 1 unit of Taq DNA polymerase (Promega), 0.8 unit of M-MLV reverse transcriptase (MBI Fermentas) and 0.8 unit RNase inhibitor (MBI Fermentas). The amplification protocol was as follows: 1 hour at 42°C for reverse transcription, 35 cycles 30 sec at 94°C, 45 sec at 55°C, 1 min at 72°C and finally 10 min at 72°C. PCR products were analysed by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide (EtBr) and visualised under UV.

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RESULTS and DISCUSSION

As a result of field inspections, PPV specific chlorotic spots and rings on leaves were observed on plum and apricot trees in Yalova, the apricot leaves from Afyon had necrotic spots and distortions, peach and nectarines from Bursa were symptomless and had smaller leaves than usual, some of the sweet cherry leaves from Afyon and Amasya had mosaic symptoms which is not specific for PPV. Analysis of the collected samples by PCR achieved that 12 out of 361 stone fruit tree samples infected by PPV (Table 2). Both neither in field observations nor in RT-PCR tests, plant samples provided from Afyon, Amasya, Burdur, Çanakkale, Isparta, and Izmir were detected as PPV infected.

The all PPV isolates detected from apricot, plum, peach and nectarine trees were tested several times by RT-PCR. In all tests, the expected band of the virus was observed in all isolates especially for plum isolates. On the other hand, RT-PCR tests of three sweet cherry trees, two from Amasya and one from Afyon provinces, one apricot tree from Afyon province, and one nectarine tree from Bursa were resulted weak bands at the same level of positive controls. In order to confirm RT-PCR results, IC-RT-PCR was used for re-testing of these isolates.

It was reported that IC-RT-PCR was proved to be 250 and 5000 times more sensitive than the RT-PCR and ELISA, respectively, for PPV detection (Wetzel et al., 1992). Despite the low titres and the uneven distribution of the virus in the infected trees, IC-RT-PCR was reported to detect the virus in most of the infected samples analysed (Wetzel et al., 1992; Nemchinov et al., 1995; Candresse et al., 1995; Rosner et al., 1998; Helguera et al., 2001). Therefore, IC-RT-PCR was applied to the two cherries from Amasya and the nectarine sample from Bursa (Figure 1a). PPV RNA's were efficiently amplified by PCR in positive control and plum sample from Yalova, but not in the cherry and nectarine samples. Negative amplifications by IC-RT-PCR were also obtained for the samples of the apricot and sweet cherry from Afyon. Consequently, it was suggested that the weak bands amplified from these samples before by RT-PCR were artefacts. Other hand, no differences were determined between used Bioreba and

Table 2. Stone fruit tree samples detected PPV infection by RT-PCR

Stone Fruit Species	Bursa	Yalova	TOTAL
Sweet cherry	0	0	0
Sour cherry	0	0	0
Peach	1	0	1
Nectarine	2	0	2
Apricot	0	2	2
Plum	0	7	7
TOTAL	3	9	12

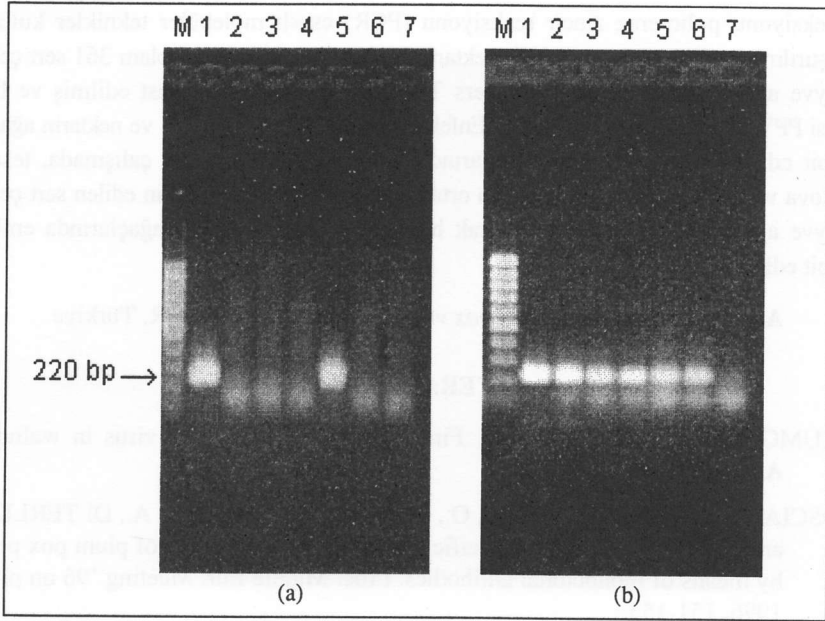


Figure 1. a) IC-RT-PCR tests of some PPV isolates. M-DNA ladder, low range (MBI Fermentas), 1- Plum from Yalova, 2- Sweet cherry1 from Amasya, 3- Sweet cherry2 from Amasya, 4- Nectarine from Bursa, 5-PPV positive control, 6-Healthy GF-305 as a negative control, 7-Negative control without RNA.

b) IC-RT-PCR with diluted sap of PPV infected leaf sample. 1-1:10, 2-1:100, 3-1:200, 4-1:300, 5-1:400, 6- 1:500, 7-Negative control without RNA.

Loewe antisera and between followed protocols for immuno-capturing. Both antisera and methods were suggested for PPV detection by IC-RT-PCR as a result of our investigation. Dilution of the samples after grinding of the samples was not necessary (Figure 1b).

PPV is considered of quarantine significance and considerable efforts such as certification schemes, eradication programs and development of rapid, sensitive and massive detection techniques are being made everywhere to limit the spread of sharka disease. The present work suggests that determination of PPV prevalence needs further investigation on, and it should be investigated in stone fruit nurseries by more advanced techniques like nested PCR and PCR-ELISA.

ÖZET

TÜRKİYE'DEKİ SERT ÇEKİRDEKLİ MEYVE AĞAÇLARINDA *Plum pox virus* (PPV)'ÜN YAYGINLIĞININ RT-PCR İLE TESPİTİ

Afyon, Amasya, Burdur, Bursa, Çanakkale, Isparta, İzmir ve Yalova bölgelerinden temin edilen sert çekirdekli meyve ağacı örneklerinde *Plum pox virus* (PPV)'ün

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enfeksiyonu polimeraz zincir reaksiyonu (PCR) esaslı moleküler teknikler kullanılarak araştırılmıştır. Kiraz, vişne, şeftali, nektarin, kayısı ve erik içeren toplam 361 sert çekirdekli meyve ağacı örneği RT-PCR (Revers Transkripsiyon-PCR) ile test edilmiş ve bunların 12'si PPV ile enfekteli bulunmuştur. Enfeksiyonlar erik, kayısı, şeftali ve nektarin ağaçlarında tespit edilmiş, kiraz ve vişne ağaçlarında tespit edilmemiştir. Bu çalışmada, test edilen Yalova ve Bursa dışında Türkiye'nin orta ve batı bölgelerinden temin edilen sert çekirdekli meyve ağaçları PPV'ünden ari olarak bulunmuş, kiraz ve vişne ağaçlarında enfeksiyon tespit edilmemiştir.

Anahtar Kelimeler: *Plum pox virus*, RT-PCR, IC-RT-PCR, Türkiye

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