ISSN 0378 - 8024

THE JOURNAL OF TURKISH PHYTOPATHOLOGY

PUBLISHED BY THE TURKISH PHYTOPATHOLOGICAL SOCIETY

Volume : 31 Number : 3 September : 2002

The Journal of Turkish Phytopathology, issued three times a year, is an official publication of The Turkish Phytopathological Society, and publishes original research papers, reports of new plant diseases and accomplishments.

Page Charges: Current charges are a $2 for each printed page for members of The Turkish Phytopathological Society and $10 for surcharge and $3 per printed page for nonmembers.

Subscription rates: $50 per year, surface postage and handling included.

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THE JOURNAL OF TURKISH PHYTOPATHOLOGY

TURKISH PHYTOPATHOLOGICAL SOCIETY

VOL. 31 September NO. 3

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First Report of Downy Leaf Spot of Walnuts Caused by *Microstroma juglandis in* Southern Turkey

Şener KURT E. Mine SOYLU Soner SOYLU F. Mehmet TOK

Department of Plant Protection, Faculty of Agriculture, University of Mustafa Kemal, 31040 Hatay / TURKEY

In the spring of 2001, infected plants of *Juglans regia* L. in Hatay province of the Mediterranean region, Turkey, showed typical downy leaf spot symptoms, especially following moist and cool climatic conditions. Initially, disease symptoms were whitish efflorescence containing fungal structures and spores, on the lower surface of the affected leaves, often concentrated along the veins, and a yellowish discoloration on the corresponding upper surface of the leaves (Figure 1). In the later stages of disease downy leaf spots enlarged, diseased tissues became necrotic, and caused a decrease in photosynthesis. Sometimes, infected walnut treeleaves were abnormal and defoliated. Examination of leaf infections using scanning electron microscopy (Figure 2) revealed whitish polygonal efflorescences which were basidial structures forming oval basidiospores (6.2 to 7.8 x 2.9 to 3.4 µm). The fungus was identified as *Microstroma juglandis* (Berenger) Sacc. (Basidiomycota, Exobasidimycetidae, Ustila-ginomycetes, Microstromatales, Microstromataceae), based on the morphological and cultural characteristics of basidium and basidial structures (Kirk et al., 2001). Isolations on potato dextrose agar (PDA) using small pieces of a surface-sterilized leaf tissue yielded a white or cream-colored, mucoid, and yeast likes colony (Figure 3) as reported by García-Jimënez et al. (1995). Microscopically, colonies consisted of ellipsoidal to

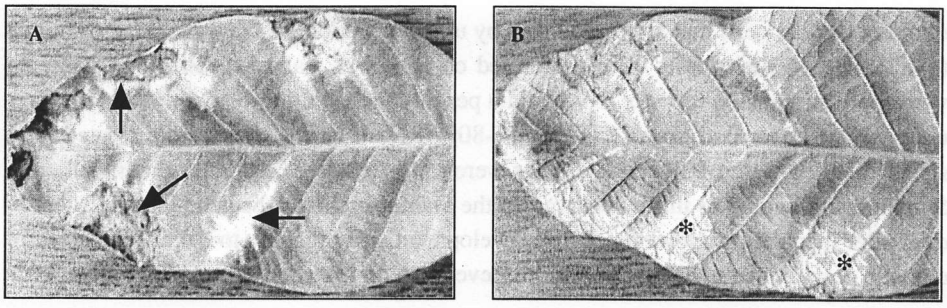

Figure 1. Typical symptoms of downy leaf spot on the upper (A) and lower surfaces (B) of the walnut leaves. A, Small arrow indicates early stage of infection showing a yellowish discoloration. Large arrows indicate the late stage of infection in which tissues becomes necrotic. B, shows typical whitish efflorescence (asterisks) containing fungal structures and spores on the lower surface of the leaf.

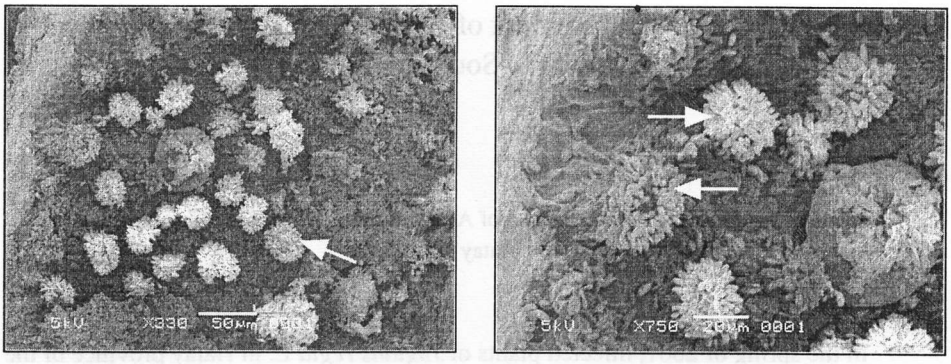

Figure 2. Electron microscopic scans of downy leaf spot on the lower surface of walnut leaves. Whitish polygonal efflorescences contain basidial structures (large arrows) forming oval basidiospores (small arrow).

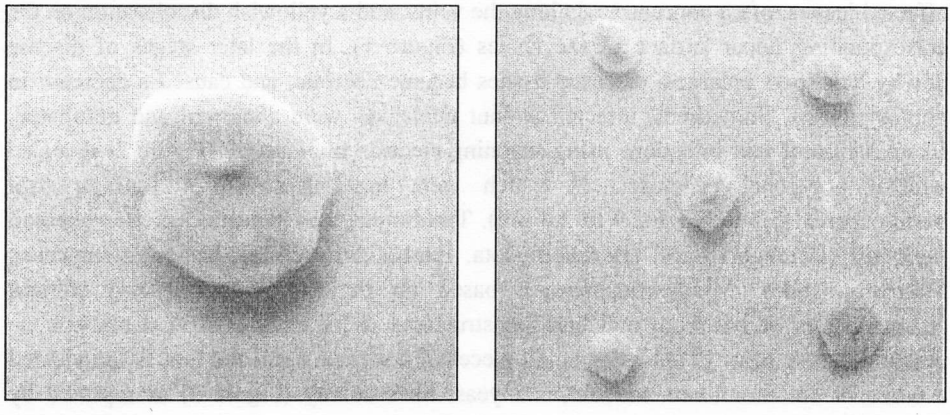

Figure 3. Cream-colored yeast-like colonies of *Microstroma juglandis* on Potato Dextrose Agar (PDA) medium.

ovoid or clavate cells that are reproduced by unipolar budding as described by Arx et al. (1982). Pathogenicity tests were performed on leaves of walnut seedlings by spraying with spore suspension (approx. 10^7 conidia per ml) and covering with polyethylene bags under the natural conditions (25±2°C, 75-80% RH). Control plants were treated with sterile distilled water. Polyethylene bags were removed three days after the inoculation. Symptoms, identical to those observed in the walnut orchards, became visible 21 to 24 days after inoculation. However, lesion development was mild due to the excessive warm climatic conditions (30±2°C). No lesions developed on leaves of the control plants.

The same fungus has been previously reported in the United States (Neely and Black, 1976), Bulgaria (Tsanova and Rosnev, 1976), India (Puttoo, 1976), New Zealand (Boesewinkel, 1977), and UK (Greig et al., 1982). However, downy leaf spots on walnuts were reported between 1993 to 1994 affecting noncultivated walnut trees in a

rainy areas of Leon, northwestern Spain, and in 1995 in Valencia province in eastern Spain, and recently in Italy (García-Jimënez et al., 1995; Anselmi, 2001). Generally the disease is not considered economically important. However, we have been informed about extensive loses from downy leaf spots in Turkey. This is the first report of downy leaf spot on walnuts caused by *Microstroma juglandis* from Hatay, Turkey.

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J. Turk. Phytopath., Vol. 31, No. 3, 117-124, 2002 ISSN 0378 - 8024

Determination of Response of Some Barley Cultivars to *Rhynchosporium secalis* Depending Upon Different Inoculation Sources and Methods

Hamit KAVAK* **Y. Zekai KATIRCIOĞLU****

* Harran University, Agriculture Faculty Department of Plant Protection, Ankara / TURKEY
** Ankara University, Agriculture Faculty Department of Plant Protection, Urfa / TURKEY

ABSTRACT

Twenty barley cultivars responded to *Rhynchosporium secalis*, causal agent of leaf blotch, with different reaction degrees depending upon inoculum sources and treatments carried out under field and controlled conditions. The sensitivity levels of cultivars were as follows; 8 resistant, 5 mean susceptible 7 susceptible under natural inoculation; 10 resistant, 7 moderate susceptible 3 susceptible at the artificial inoculation in the field treatments; 7 resistant, 6 moderate susceptible and 7 susceptible at the artificial inoculation in controlled room treatments. In addition, there was three levels of resistance on cultivars in this classification. On the other hand, with the highest infection rates, Lenka was the most susceptible cultivar in the all treatments, while W 1936 was the most resistant cultivar. This study was carried out at treatment fields of Agricultural Faculty, Harran University in Sanlıurfa under arid conditions of Turkey.

Key words: Barley, *Rhynchosporium,* Inoculation Sources

INTRODUCTION

With the 252.000 hectare fields, Şanlıurfa has the second most wide of barley farm lands in Turkey (Anonymous, 1999). This also within South Eastern Anatolia District which is the most arid climate of Turkey. Almost of the rainfall occurs between November and April or mid-April as rain. Thus, all cereal sowings should be carried out in November. Winters are generally warm and at the some days quite cold. On the other hand, the rainfall amount in some years much more increase than long term average of district. Therefore, this climate can permit to development of scald disease of barley at the epidemic levels when susceptible hosts are shown and suitable climate occurred. This disease was detected for the first time in 1995 in Sanlıurfa barley fields, and surveyed at 12 administrative district during 1996 (Kavak and Katırcıoğlu, 1999).

Barley cultivars exhibit different reaction to barley leaf blotch, caused by *Rhynchosporium secalis*; while some cultivars can become resistant, others susceptible according to the forms of reaction. In addition, reaction degrees vary depending upon some factors, including sowing time, growth stage of crops at inoculation time, the

situation of inoculum sources and climatic conditions etc. (Habgood, 1975; Bockelman et al., 1977; Döken 1979; Karjalainen, 1990; Arma et al., 1991; Xue and Hall, 1991; Xue et al., 1994.

To supply the malt requirements of Turkey, cultivars used in this study, are generally sown in Mean Anatolian District, Trakya District and some locations of the Mediterranean District of Turkey (Başgül and Engin, 1995).

The aim of this study was to determine reactions of 20 barley cultivars against leaf blotch disease under arid conditions of Turkey using different inoculum sources and methods.

MATERIALS and METHODS

Fungal material: Infected straws derived from an infected barley field and mixed spore populations which were derived from 60 isolates and produced on LBA (Lima Been Agar) formed the main fungal materials.

Plant material: 20 barley cultivars used in experiments formed main plant material of this study and the origin of them are in these respects: Efes 3, Efes 4 Efes 6 Efes 8 Efes 9 and Kaya are Turkey; Alexis, Angora, Berolina Cristin, Hege-1244, Lenka, S448/88,W 12474, W 9026, W1936 are German; Blenhaim is England; Stirling and Schooner are Australia; ND8972 is USA (Başgül and Engin 1995).

Sample collection: The artificial inoculum source was a spore population derived from pathogen isolates belonging to Sanlıurfa centre and its 11 administrative counties. During the vegetative period of 1996, five samples collected per districts and derived an isolate per sample. All isolates were then mixed to form spore population and applied to experiments.

Isolation: Samples bearing the lesion excised from infected leaves were surface sterilised for 10s in 70% ethyl alcohol and for 90s in 0.5% NaOCI, rinsed in sterile distilled water, placed onto petri dish containing moist filter paper to encourage the sporulation for 24h. To release of spores into the water, samples in sterile distilled water were rinsed vigorously A drop of suspension, including *R. secalis* spores, was then spread over the surface of water agar (WA) plate, and spores were germinated (Fowler and Oven, 1971). Germinating spores were transferred on Lima been agar plates (LBA) containing 0.1 ml of lactic acid in each petri dish, with a sharp pointed platinum needle, and incubated at 17 °C for 15 days to form of colony. Sixty colonies were derived from sixty infected samples, as a colony per sample. Pure isolates of pathogen were produced by applying each colony to fresh LBA, and incubating described as above. Pathogen was produced on LBA at 17 °C during experiments (Ayesu-Offei and Clare, 1970; Ali, 1975). To prepare the mixed spore suspension, some water was added to each plate and their aqueous suspensions poured to a big Erlenmeyer one by one after scrapping from the

surface of the petri dish with a microscope slide. The mixed suspension was then diluted to give 1.2x106 spore per ml later filtered through one layer of muslin.

Experimental design and treatments: Sowings were carried out on 14 November as suitable of climatic condition of district. Field experiments were four replicates, randomised blocks and 1.2 m x 5 m, and seeds were sown as 500/seed per m^2. Climatic room treatments were three replicates, and dark polyethylene bags, 13 cm x 25 cm, were used in addition to raised four plants per pot. Seeds were kept at 1±1 °C in the refrigerator for 30 days before sowing. The light intensity of climatic room was 15000 lux, and plants were raised at 16h/light and 8h/dark period. The temperatures of climatic room was 18-22 °C in the light and 14 -18 °C in the dark period.

Inoculation technique: By establishing on an infected field, the experiments in 1996 was inoculated naturally as well as by spreading infected barley straws in plots at growth stage (GS) 11. The first inoculation for experiments in 1997 was also made at GS 12, by spraying $1.2x10^6$ spore per ml suspension of *R. secalis* in addition to other inoculations applied at growth stages 29 and 51 (Zadoks et al. 1974). Inoculations were applied in evening when the weather was cloudy and relative humidity was high. When relative humidity was low after inoculation, plots were sprayed with water without run off on leaves two times a day during 48 hour. Pots in the climatic room were kept at high humidity (95% - 100%) for 48 hour after inoculation, and then transferred to normal situation. Pots were watered when needed, as surface watering.

Assessments of Disease Index: Reaction degrees exhibited by cultivars were measured between growth stage 75 and 85 estimating the percentage area infected (James et al., 1968) on each of the top four leaves of a random sample of 25 tillers in each plot. To convert the disease assessments on individual leaf positions into overall plots infection levels, measurements were made on the upper four leaves of 25 tillers drawn at random from each plot at growth stage 85; plot infection levels were calculated as Disease Index (DI) (Zhang et al., 1987). ($DI=\sum ai/\sum a$ where a is the mean area of each leaf position and i its mean infection level). Then, in order to test DI values for possible significant differences, the Duncan multiple range test, was used (P=0.05). The resistance levels of cultivars were classified according to 0-4 scale (Lyngs Jorgensen, 1992) where: 0-2 resistant, 3 mean susceptible, 4-5 susceptible.

RESULTS and DISCUSSION

In the field experiments under natural conditions in 1996, twenty barley cultivars presented different reaction against leaf blotch. The cultivar W 1936 was the most resistant one and it didn't form any symptom. Lenka was the most susceptible cultivar with 66.2% infection rate. Other cultivars were showed different symptom rates between two numbers. The reaction degrees of cultivars were statistically significant (P=0.05), (Table 1).

DETERMINATION OF RESPONSE OF SOME BARLEY CULTIVARS TO *RHYNCHOSPORIUM SECALIS* DEPENDING UPON DIFFERENT INOCULATION SOURCES AND METHODS

Table 1. Reaction degrees and their groups of 20 barley cultivars to *R. secalis*, based on treatments

		Treatments					
		1		2		3	
No	Cultivars	*RD	**G	*RD	**G	*RD	**G
1	Efes 9	10.8	g	7.8	f	11.2	ı
2	Efes 4	8	gh	5.6	fg	9.1	ı
3	Efes 6	9.6	g	6.8	fg	10.5	ı
4	Efes 8	10	g	7.4	f	10.7	ı
5	Blenhaim	62.6	a	55.8	a	63.4	b
6	Alexis	52.8	b	46.4	b	57.3	c
7	W 12474	2.2	ıi	1.2	ıi	2.8	j
8	W 9026	2	ıi	1.3	ıi	2.1	jk
9	W 1936	0	i	0	i	0	k
10	S 448/88	4.8	hı	2.9	hı	5.6	i
11	Stirling	36.4	e	30.2	d	39	f
12	Hege-1246	46.2	cd	39.2	c	50.3	d
13	Lenka	66.2	a	58.2	a	69.2	a
14	Schooner	44.2	cd	38.4	c	49.5	d
15	ND 8972	24.8	f	19.6	e	26.8	ıg
16	Cristin	43.2	d	37.8	c	47.0	e
17	Berolina	47.4	c	38.6	c	50.7	d
18	Efes 3	8.6	g	5.1	gh	9.8	ı
19	Kaya	22	f	18.1	e	24.3	h
20	Angora	2	ıi	1.2	ıi	2.4	j

1=Natural inoculation 2=Artıficial inoculation (Field) 3=Artıficial inoculation (Climatic room) *=Reaction degrees % Disease Index (DI) **=Duncan grouping. Values followed by different letters in a column are significantly different (P=0.05).

The artificial inoculation, applied to field and controlled room experiments in 1997, 20 barley cultivars exhibited reaction at different rates. At field plots, W 1936 did not showed any symptom. But lesions were detected on leaves of this cultivar in pots in the controlled room with an infection rate (average 0.4mm diameter, dark spot), as well as most resistant cultivar. The most susceptible cultivar was Lenka, and its infection rate was 58.2% at field treatments and 69.2% in controlled room experiments, respectively. Also, other cultivars expressed different reactions between two cultivars, and these were grouped into 11 in the field and 12 in the controlled room experiments (P=0.05) (Table 1, Treatments 2 and 3).

Reaction degrees exhibited by cultivars to *R secalis* varied, depending upon treatments carried out in different conditions. For example, at the natural inoculation (Treatment 1 in

Table 2) seven cultivars exhibited the reaction degree at 4th scale, while in the artificial inoculation (Treatment 2 in Table 2), only three cultivars could show reaction degree at the same scale. In addition to these susceptible cultivars described, similar results occurred on resistant or mean susceptible cultivars (Treatment 1, 2, 3 in Table 2). When considering all treatments, the sensitivity levels of 20 cultivars are presented as follows according to their infection rates (Table 2).

Table 2. Groups of sensitivity levels of cultivars exhibiting different degrees of reaction to *R. secalis**

		Treatments					
		1		2		3	
No	Cultivars	RD	DR	RD	DR	RD	DR
1	Efes 9	10.8	3	7.8	2	11.2	3
2	Efes 4	8	2	5.6	2	9.1	2
3	Efes 6	9.6	2	6.8	2	10.5	3
4	Efes 8	10	3	7.4	2	10.7	3
5	Blenhaim	62.6	4	55.8	4	63.4	4
6	Alexis	52.8	4	46.4	4	57.3	4
7	W 12474	2.2	1	1.2	1	2.8	1
8	W 9026	2	1	1.3	1	2.1	1
9	W 1936	0	0	0	0	0	0
10	S 448/88	4.8	1	2.9	1	5.6	2
11	Stirling	36.4	3	30.2	3	39	3
12	Hege-124	46.2	4	39.2	3	50.3	4
13	Lenka	66.2	4	58.2	4	69.2	4
14	Schooner	44.2	4	38.4	3	49.5	4
15	ND 8972	24.8	3	19.6	3	26.8	3
16	Cristin	43.2	4	37.8	3	47.0	4
17	Berolina	47.4	4	38.6	3	50.7	4
18	Efes 3	8.6	2	5.1	1	9.8	2
19	Kaya	22	3	18.1	3	24.3	3
20	Angora	2	1	1.2	1	2.4	1

RD = Reaction degrees % or DI values

*DR = Disease degrees, according to Lyngs Jorgensen (1992)

There was some factors limiting the infection development on cultivars between years and treatments.

Experiments in 1996 were established on an infected barley field and inoculated by straw inoculum at second time at G.S. 11 and 12. Therefore, conditions conducted here was almost the same of farm conditions, and epidemics were normally initiated much earlier from inoculum borne on stubble as pointed out by authors (Skoropad 1966 and

Habgood 1975). Because, there were enough inoculum source on infected straw debris to initiate the primary infection (Polley, 1971). In addition, adequate inoculum was stored at lower leaves to infect the upper leaves (Skoropad 1966, Habgood, 1975). In contrast to previous year, due to constituted on a site without previous cereals, field experiments in 1997 were conducted somewhat under artificial conditions. Spore inoculation, applied to seedlings at GS. 11 and 12, did not achieve sufficiently to start the primary infection due to limiting factors. Because, the seedlings were very small and the temperature at night was between 2 °C - 5 °C when inoculation were applied. So, it is estimated that artificial inoculation carried out in field experiments in 1997 was not as effective as natural inoculum sources in 1996 when considering that the dissemination of inoculum between crops is very limited (Ayesu-Offei and Carter, 1971) and also, low temperature at inoculation time limits the infection success of leaf blotch on leaves (Caldwel, 1937 and Shaner, 1981.

Because of used much more isolates, higher reaction degrees of cultivars may expect in 1997 experiments compared to 1996 ones. But this was not occurred due to variable of climatic conditions between two years. However, the virulence degree of pathogens merits detailed research using test plants for each isolate.

As a result; cultivars exhibiting reaction degrees at 1and 2 scales, are considered to be reliable for sowing under arid conditions, while mean susceptible cultivars with scale of 3 considered reliable only in years that have less rainy. Other cultivars with scale 4 are generally agreed susceptible. Thus, some control methods should be considered in the case of sown this cultivars.

ÖZET

FARKLI İNOKULUM KAYNAKLARI VE METODLARA BAĞLI OLARAK BAZI ARPA ÇEŞİTLERİNİN *RHYNCHOSPORIUM SECALIS*'E KARŞI REAKSİYONLARININ BELİRLENMESİ

Tarla ve kontrollu koşullarda, doğal ve yapay inokulum kaynaklarının kullanılarak yürütülen çalışmalarda 20 arpa çeşidi arpa yaprak lekesi etmeni, *Rhynchosporium secalis*'e karşı aşağıdaki şekilde farklı reaksiyon göstermiştir. Doğal inokulasyonda 8 dayanıklı, 5 orta dayanıklı, 7 hassas; yapay inokulasyonunun kullanıldığı tarla denemelerinde 10 dayanıklı, 7 orta hassas, 3 hassas; yapay inokulasyonun kullanıldığı kontrollu denemelerde 7 dayanıklı, 6 orta dayanıklı ve 7 hassass. Ayrıca bu sınıflandırmada dayanıklık derecesinin üç farklı seviyesi vardı. Bütün denemelerde, en yüksek hastalık seviyesi ile, Lenka en hassas çeşidi oluştururken, W 1936 en dayanıklı çeşidi teşkil etmiştir. Çalışma, Türkiye'nin en kurak bölgesinde, Harran Üniversitesi Ziraat Fakültesi deneme alanlarında yürütülmüştür.

Anahtar Kelimeler: Arpa, *Rhynchosporium*, İnokulum Kaynakları

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J. Turk. Phytopath., Vol. 31, No. 3, 125-136, 2002 ISSN 0378 - 8024

Effect of Nitrogen, Potassium, High Relative Humidity and Low Night Temperatures on The Incidence of Pith Necrosis of Tomatoes

N. ÜSTÜN* **H. SAYGILI****

* Plant Protection Research Institute, 35040 Bornova / İZMİR

** Ege University, Faculty of Agriculture, Department of Plant Protection 35100 Bornova / İZMİR / TURKEY

ABSTRACT

In four separate greenhouse experiments one month old tomato plants grown in pots were artifficially inoculated with 10^8 CFU/ml bacterial suspension of *Pseudomonas cichorii* (PC4), *Pseudomonas viridiflava* (PV3) and *Erwinia carotovora* subsp. *carotovora* (EC2). Nitrogen applied as nitrate and ammonium at 0, 150, 300 and 450 ppm levels affected the plant growth with some phytotoxic effect at high levels. All nitrate and 150 ppm ammonium nitrogen levels increased PV3 and EC2 infections, but 300 and 450 ppm ammonium levels had no effect on disease development. Nitrogen treatments (except 150 ppm nitrate-nitrogen) decreased disease severity of PC4. Potassium applications (0, 200, 400, 600 ppm) reduced disease incidence and there was negative correlation between disease development and potassium levels. High relative humidity increased the length of necrosis, caused by PC4, PV3 and EC2 at the rate of 22.17%, 23.33% and 42.59%, respectively. Low night temperature enhanced infection by PC4 and EC2 (15.17% for PC4 and 37.44% for EC2), but did not influence significantly (P = 0.05) the incidence of PV3. High relative humidity in combination with low night temperature increased disease severity caused by PC4 and EC2 (42.52% and 42.38%, respectively), but had no effect on PV3 infection. Nitrogen level, causing highest disease severity, potassium deficiency, high relative humidity and low night temperature in combination increased length of necrosis by PC4, PV3 and EC2 53.89%, 54.65% and 48.99%, respectively.

Key words: Tomato pith necrosis, nitrogen and potassium fertilizers, relative humidity, temperature

INTRODUCTION

Pith necrosis of greenhouse-grown tomatoes have been observed in Aegean Region of Turkey since 1988 (Demir, 1990). In recent years, the prevalence of the disease has increased between 20-30% (Üstün, 2000) causing sometimes considerable losses. It is known that the disease is caused by low-grade pathogens such as *P. corrugata* Roberts and Scarlett 1981, *P. cichorii* (Swingle 1925) Skapp 1928, *P. viridiflava* (Burkholder

1930) Dowson 1939, *Pseudomonas* sp., *E. carotovora* subsp. carotovora (Jones 1901) Bergey, Harrison, Breed, Hammer and Huntoon 1923, *E. carotovora* subsp. *atroseptica* (van Hall 1902) Dye 1969 and/or *E. chrysanthemi* Burkholder, Mc Fadden and Dirnock 1953 (Wilkie et al., 1973, Wilkie and Dye, 1974, Scarlett et al., 1978, Alivizatos, 1986, Malathrakis and Goumas, 1987, Dhanvantari, 1990, Wick and Shrier, 1990). In our studies, however, *P. cichorii, P. viridiflava* and *E. carotovora* subsp. *carotovora* were the predominate bacteria isolated from diseased plants (Üstün, 2000, Üstün and Saygılı, 2001). Certain environmental conditions may predispose tomato plants to invasion by low-grade pathogens. For example, nitrogen fertilization, high relative humidity and low night temperature have been observed to influence development of pith necrosis (Scarlett et al., 1978, Alivizatos, 1986). The objectives of this investigation were to study the effect of (i) nitrat and ammonium forms of nitrogen; (ii) potassium; (iii) high relative humidity and low night temperature (iv) combination of the factors mentioned above on disease severity, incited by *P. cichorii, P. viridiflava* and *E. carotovora* subsp. *carotovora* under greenhouse conditions, experimentally.

MATERIALS and METHODS

Preparation of Plant Materyal: Four separate greenhouse experiments were carried out in autumn-spring seasons of 1998 and 1999. Ten-day old tomato seedlings of the variety Fantastic F 144 RN were transplanted into plastic pots (220x200 mm) containing 4 kg of air dried soil with very low nutrient content (Mn - 1.6 ppm, Cu - 0.1 ppm, Fe - 1.3 ppm, Zn - 0.3 ppm, Mg - 160 ppm, P - 3 ppm, K - 110 ppm, Na - 20 ppm, Ca - 2800 ppm, total N- 0.036%, organic matter- 072%, lime- 3.78%, salt <0.3 ppm). The soil structure was sandy loam (sand - 63%, mile - 28.92%, clay - 7.48%) and the NO_3 and $(NH_4)_2SO_4$ for N, Triple Superphosphate (TPS) for, KH_2PO_4 for K and P. Fertilizers were thoroughly mixed with the soil prior to transplanting and pots with soil were watered with 810 ml water to give the soil a 60% of water holding capacity. Througout the experiments the soil moisture was maintained at 60% of water holding capacity of the soil.

Inoculum Preparation and Inoculatıon Method: One month after transplanting plants were inoculated with bacterial suspension of strains identified as *P. cichorii* (PC4), *P. viridiflava* (PV3) and *E. carotovora* subsp. *carotovora* (EC2), previously (Üstün and Saygılı, 2001). The bacterial strains were grown at 25 °C on SNA (Hayward, 1960) for 24 hours. Bacterial growth were than suspended in sterile distilled water and adjusted to 10^8 CFU/ml using a spectrophotometer. Each plant was inoculated at two levels (10 cm and 30 cm from the soil) by injecting of 0.5 ml of bacterial suspension into stem at leaf bases as described by Lopez et al. (1994). Following inoculation tomato plants were covered separately with a clear polyethylene bags for 72 hours. Length of pith necrosis was recorded one month after inoculation. Statistical analysis was performed by TARİST computer program developed by Faculty of Agriculture, Ege University (Açıkgöz et al., 1994).

Effect of nitrogen: Two different forms of nitrogen (nitrate and ammonium) at four rates (0, 150, 300 and 450 ppm) were used. For this experiment, the rates of phosphor (150 ppm) and potassium (400 ppm) were kept constant. Treatments included two nitrogen forms at rates each, and four-inoculation treatment (inoculation with PC4, PV3, EC2 and sterilized water control). Treatments were replicated three times and pots were arranged on a greenhouse bench using a factorial randomized plots design. The greenhouse temperature ranged from 18 -25 °C and relative humidity from 50-70% during the experiment. Nitrogen levels of mature leaves were determined using Modified Kjeldahl Method (Kacar, 1972). Phosphorous and potassium levels were determined as previously described (Lott et al., 1956, Kacar, 1962, Kacar, 1972).

Effect of potassium: Four rates of potassium (0, 200, 400, 600 ppm) were tested. The rates of nitrogen (150 ppm) and phosphor (150 ppm) were kept constant. Experiments were arranged in a factorial randomized plot design which included four potassium rates and four inoculation treatments (inoculation with PC4, PV3, EC2 and sterilized water control). Treatments were replicated 4 times. Tissue contents of potassium levels were determined as previously described (Lott et al., 1956, Kacar, 1962, Kacar, 1972).

Effect of high relative humidity and low night temperature: The experiment was conducted in three separate chambers in the greenhouse. Tomato plants were transplanted in pot soil containing 150 ppm nitrogen, 150 ppm phosphor and 400 ppm potassium. Plants were kept at 18-25 °C. The relative humidity in the greenhouse varied between 50% and 70%. Plants were inoculated with PC4, PV3 or EC2 as previously described. Plants inoculated with sterile water served as control. After inoculation, in control chamber plants were grown at temperatures where minimum ranged between 13-19 °C and maximum between 22-32 °C and relative humidity ranged between 50-70%. In second chamber studied the effect of high relative humidity the inoculated plants were kept at 88-92 % high relative humidity and at same temperatures as in control chamber. In the third chamber in which the effect of low night temperature studied plants were kept at temperatures where minimum ranged between 0 to 11 °C and maximum ranged between 14 to 29 °C and at same relative humidity conditions as in control chamber. The experiment was arranged in a factorial randomized plot design and included three different conditions (high relative humidity; low night temperature and control) and four inoculation.

Effect of combination of nitrogen, potassium, high relative humidity and low night temperature. The experiment was conducted in two separate chambers in the greenhouse. In one of the chambers the tomato plants were transplanted in soil containing 150 ppm nitrogen, 150 ppm phosphor and 400 ppm potassium and grown at 18-25 °C with 50-80% relative humidity (control chamber). In the second chamber, the effect of combination of the above factors was investigated. The plants were transplanted in pots containing 150 ppm nitrogen, 150 ppm phosphor and 400 ppm potassium or in soil containing 150 ppm nitrogen, 150 ppm phosphor and 0 ppm potassium. Before

inoculation, plants were grown at same temperature and relative humidity conditions as in the control chamber. After inoculation with strains PC4, PV3 , EC2 or sterile water, plants were kept under high relative humidity (88-92%) and low night temperature (7-12 °C). The experiment was arranged in factorial randomized plot design and conducted under three different conditions: (i) 150-ppm nitrogen, 150-ppm phosphor, 400-ppm potassium + 50-80% relative humidity + temperatures with minimum of 15-20 °C and maximum of 22-35 °C; (ii) 150-ppm nitrogen, 150-ppm phosphor, 400-ppm potassium + 88-92% relative humidity + low night temperatures with minimum ranging between 7-12 °C and maximum ranging between 18-32 °C; and (iii) 150-ppm nitrogen, 150-ppm phosphor, 0-ppm potassium + high relative humidity range of 88 to 92% + low night temperatures that ranged between 7-12 °C.

RESULTS and DISCUSSION

Effect of nitrogen: Nitrogen applications influenced plant growth, significantly (Table 1). The best growth was obtained at 150 - ppm of nitrogen level supplied as nitrate or ammonium. The plants generally grew better in soil treated with nitrate than the ammonium rates. Regardless of the nitrogen form plant showed phytotoxicity at 300 and 400 ppm nitrogen levels 5 to 6 days following transplanting. Plant growth was greatly reduced (Table 1). High rates of nitrate caused unusual burning spots on the leaves and

Table 1. The effect of various N applications on plant growth, nutrient content of tissue (% dry weight) and length of pith necrosis

N forms	N dose (ppm)	Plant Growth (cm)		Mean % of dry weight leaf tissue					Length of pith necrosis (cm)		
		Plant height	Stem diameter	N	P	K	Ca	Mg	PC4	PV3	EC2
NO3-N	0	75.33	0.55	1.17	0.14	1.60	2.2	0.44	17.46	2.27	2.93
	150	87.00	0.83	1.82	0.16	2.14	2.4	0.4	18.21	9.46	14.17
	300	82.83	0.64	1.93	0.17	1.88	2.0	0.36	6.96	7.49	8.12
	450	64.66	0.57	2.94	0.13	1.78	3.3	0.36	11.75	4.61	9.42
Significance	Linear	**	Ns	LSD (P=0.05) = 0. 372					**	*	**
	Quadratic	**	**						**	**	**
NH4-N	0	75.33	0.55	1.17	0.14	1.60	2.2	0.44	17.46	2.27	2.93
	150	83.83	0.71	1.79	0.16	1.60	2.0	0.31	11.89	8.47	11.97
	300	57.67	0.61	1.76	0.16	1.88	2.0	0.31	6.03	2.03	2.28
	450	48.83	0.49	3.24	0.15	1.98	2.8	0.36	2.33	1.64	2.28
Significance	Linear	**	*	LSD (P=0.05) = 0. 372					**	**	**
	Quadratic	**	**						Ns	**	**

* statistical significance at P= 0.05, ** statistical significance at P= 0.01, ns= not significant, PC4- *Pseudomonas cichorii*, PV 3- *Pseudomonas viridiflava*, EC 2- *Erwinia carotovora* subsp. *carotovora*

high rates of ammonium induced wilting of plants. Phytotoxicity effect increased with increasing of nitrogen rates and was more severe at high ammonium levels than the high nitrate levels (Fig. 1).

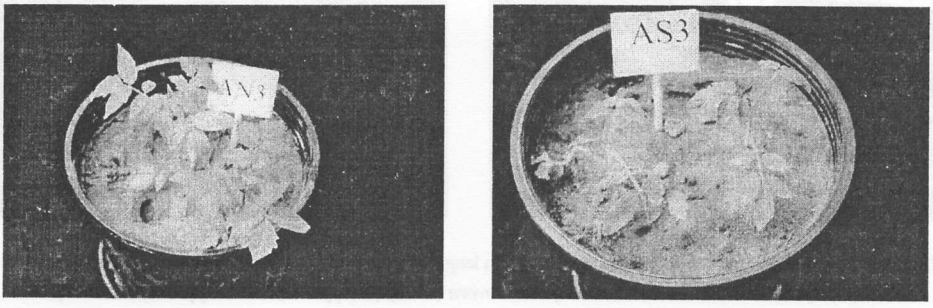

Figure 1. Phytotoxicity symptoms (AN 3- 450 ppm nitrate- nitrojen; AS 3-450 ppm ammonium –nitrojen).

All nitrate and 150 ppm ammonium levels increased PV3 and EC2 infection, while other ammonium levels did not affect disease development by PV3 and EC2 infection. On the other hand, with the exception of 150-ppm nitrate rate, all nitrogen treatments significantly (P=0.05) decreased disease severity of PC4 (Table 1).

Effect of potassium: Potassium levels did not affect plant growth significantly (P=0.05). However, potassium tissue content increased with increasing of K levels (Table 2). Increases in K levels generally, resulted in reduced incidence of pith necrosis caused by PC4, PV3 and EC 2 (Table 2). Results of regression analyses showed significant negative correlation between potassium levels and disease severity. Regression equations for potassium and disease severity for PC4, PV3 and EC2 were $Y = 7\ E - 05\ x^2 - 0.0554\ x + 24.467$ ($R^2 = 0.97$); $Y = 6\ E - 05\ x^2 - 0.056\ x + 17.602$ ($R^2 = 0.997$) and $Y = 3\ E - 05\ x^2 - 0.0499\ x + 21.532$ ($R^2 = 0.949$), respectively.

Table 2. The effect of various K applications on plant growth, tissue nutrient content (% dry weight) and length of pith necrosis

K rate (ppm)	Plant growth (cm)		Mean % of dry weight leaf tissue					Length of pith necrosis (cm)		
	Plant height	Plant thickness	N	P	K	Ca	Mg	PC4	PV3	EC2
0	106.98	0.61	1.79	0.16	1.54	2.2	0.35	24.758	17.710	20.75
200	114.1	0.65	1.65	0.18	1.62	2.0	0.34	15.175	8.708	15.208
400	106.17	0.63	2.07	0.18	1.64	2.0	0.31	14.144	5.875	4.500
600	112.93	0.63	2.94	0.15	2.02	2.8	0.35	15.413	7.243	4.250
Significance										
Linear	Ns	Ns	LSD (P =0.05) = 0.385					**	**	**
Quadratic	Ns	Ns						**	**	**

* statistical significance at P= 0.05, ** statistical significance at P= 0.01, ns=not significant, PC4- *Pseudomonas cichorii*, PV 3- *Pseudomonas viridiflava*, EC 2- *Erwinia carotovora* subsp. *carotovora*

Figure 2. showed the effect of potassium applications on length of pith necrosis.

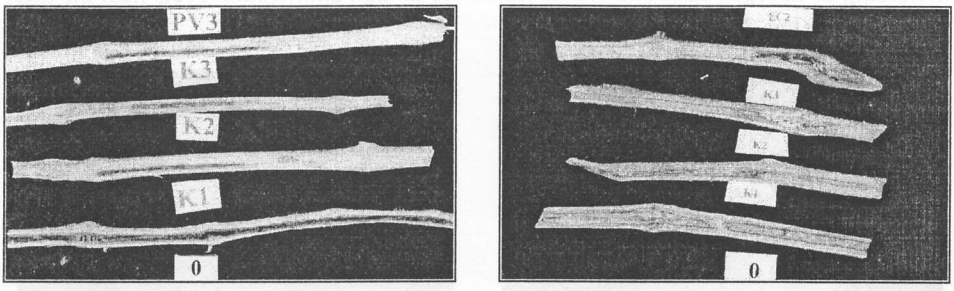

Figure 2. The effect of various potassium aplications on length of pitn necrosis caused by *Pseudomonas viridiflasva* (PV3) and *Erwinia carotovora* subsp. *carotovora* (EC 2) (0-0 ppm K; K1-200 ppm K; K2- 400 ppm K; K 3- 600 ppm K).

Effect of high relative humidity and low night temperature: High relative humidity increased the length of pith necrosis by PC4, PV3 and EC2 at the rate of 22.17%, 23.33% and 42.59%, respectively (Table 3). Low night temperature enhanced infection by PC4 and EC 2 (15.17% for PC 4 and 37.44% for EC2), but not influenced PV 3 significantly (Table 3).

Table 3. The effect of High relative humidity and Low night temperature on disease severity by PC4, PV3 and EC2

Isolate	Length of pith necrosis (cm)			Increases in length of pith necrosis (%)	
	Control	HRH	LNT	HRH	LNT
PC 4	13.448	16.442	15.500	22.17	15.17
PV 3	5.527	6.817	5.850	23.33	5.844
EC 2	6.692	9.970	9.610	42.59	37.44

LSD (P= 0.05)= 1.264 HRH= high relative humidity, LNT= low night temperature, PC 4- *Pseudomonas cichorii*, PV 3- *Pseudomonas viridiflava*, EC 2- *Erwinia carotovora* subsp. *carotovora*

The Effect of Combination of N, K, High Relative Humidity and Low Night Temperature. When inoculated plants was kept under high relative humidity and low night temperature there was an increase of 42.52% and 42.38% in length of pith necrosis caused by PC4 and EC2, respectively. The disease increase was higher when the parameters studied were combined (Table 4).

The increase in the length of pith necrosis due to high relative humidity, low night temperature and combination of these factors are shown more clearly in Fig. 3.

Figure 3 shows that high relative humidity increased disease severity more than low night temperature. Combination of high relative humidity and low night temperature caused cumulative effect on PC4, influenced EC2 at the same rate with high relative humidity, but did not affect disease severity by PV3. Also, disease severity of all pathogens studied

Table 4. The effect of combination of N, K, High Relative Humidity and Low Night Temperature

Isolate	Length of pith necrosis (cm)			Increases in length of pith necrosis (%)	
	A	B	C	A	B
PC 4	15.917	17.187	11.168	42.52	53.89
PV 3	3.050	5.849	3.782	-	54.65
EC 2	12.28	12.827	8.623	42.38	48.99

LSD (%5) =1.307; A = (150 ppm N + 150 ppm P + 400 ppm K) + high relative humidity + low night temperature; B = (150 ppm N + 150 ppm P + 0 ppm K) + high relative humidity (88-92%)+ low night temperature (t min = 0-11°, t max = 14-25 °C); C = (150 ppm N + 150 ppm P +400 ppm K) + 50-70% relative humidity + temperature (t min = 13-19 °C, t max = 14-25 °C) PC 4- *Pseudomonas cichorii*, PV 3- *Pseudomonas viridiflava*, EC2- *Erwinia carotovora* subsp. *carotovora*

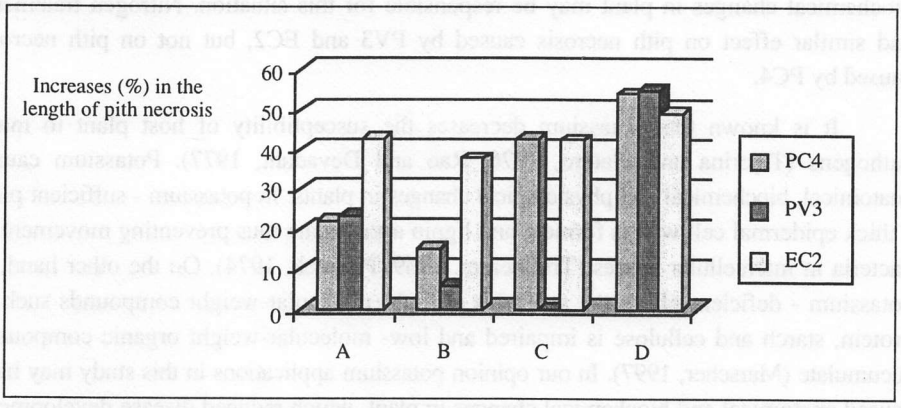

Figure 3. Increases (%) in length of pith necrosis by High Relative Humidity (A), Low Night Temperature (B), Combination of High Relative Humidity and Low Night Temperature (C) and Combination of N, K, High Relative Humidity and Low Night Temperature (D), PC4- *Pseudomonas cichorii*, PV3- *Pseudomonas viridiflava*, EC2- *Erwinia carotovora* subsp. *carotovora*.

was the highest in plants grown in pots fertilized with nitrogen at 150 ppm and potassium at 0 ppm rates when kept under high relative humidity and low night temperature regime.

Mineral nutrition has been shown to have varying affects on the host-bacterial pathogen relationship. With some host-pathogen relationships, increased nitrogen rates show increased disease susceptibility (Haygood et al., 1982, Carroll et al., 1992), whereas with others an inverse relationship exist (Marko and Stall,1983, Jones et al., 1988).

Nitrogen probably influences severity of tomato pith necrosis disease indirectly by its influence on plant growth and succulence. It has been reported that succulent and fast-growing plants are more susceptible to pith necrosis than slow-growing plants (Scarlett et al., 1978, Carroll et al., 1992). In our study disease severity by the test pathogens was the

highest at rate and nitrogen forms, in which maximum vegetative growth of plants was provided. For example, best growth of plants and highest disease severity were at 150 ppm rate of nitrogen (Table 1). Based on the results obtained, we concluded that nitrogen forms that promote vegetative growth of plants increases disease severity of pith necrosis caused by PC4, PV3 and EC2. High rates of nitrogen (300 and 450 ppm), especially NH_4-N greatly reduced plant growth due to phytotoxicity. Although phytotoxic levels showed trend of decrease in disease severity by the pathogens, disease severity by PV3 and EC 2 at 300 and 450 ppm nitrate levels was higher in comparison with 0 ppm nitrate and was not statistically significant at 300 and 450 ppm of NH_4-N. However, nitrogen treatments (except 150 ppm NO_3-N) decreased disease severity caused by PC4. Under this experimental circumstances nitrate nitrogen treatments at 300 ppm seems to be more efficient than 450 ppm in terms of suppress the disease development by PC4. Possible biochemical changes in plant may be responsible for this situation. Nitrogen treatments had similar effect on pith necrosis caused by PV3 and EC2, but not on pith necrosis caused by PC4.

It is known that potassium decreases the susceptibility of host plant to many pathogens (Tejerina and Platero, 1976, Rao and Devadaht, 1977). Potassium causes anatomical, biochemical and physiological changes in plants. In potassium - sufficient plant a thick epidermal cell wall is formed, and lignin accumulate thus preventing movement of bacteria in intercellular spaces (Trolldenier, 1969, Pissarek, 1974). On the other hand, in potassium - deficient plants the synthesis of high- molecular-weight compounds such as protein, starch and cellulose is impaired and low- molecular-weight organic compounds accumulate (Marscher, 1997). In our opinion potassium applications in this study may have caused anatomical and biochemical changes in plant, which reduced disease development. Alivizatos (1986) reported that in a hard, wooden stem with hard and limited pith the disease progress is slow and disease development is minimal. Our results support the results of Dvantvantari and Papadopoulos (1995) who reported the suppression of bacterial stem rot caused by *E. carotovora* subsp. *carotovora* in hydroponically grown tomato plants where the nutrient solution contained high potassium/nitrogen ratios.

High relative humidity and low night temperature were found to be some of the factors favored tomato pith necrosis development (Scarlett et al., 1978) These factors influence plant succulence and predispose the plants to pith necrosis pathogens. Scarlett et al. (1978) reported that the disease has most frequently occurs in unheated tomato crops where plant growth is soft due to low night temperature coupled with high nitrogen levels in the soil. High relative humidity and low night temperature increased disease severity by PC4 and EC2. In our studies, PV3 infections were influenced by high relative humidity, but were not affected by low night temperatures. High relative humidity increased disease severity more than low night temperature. In previous studies (McFadden, 1961, Jones et al., 1984) it was shown that temperatures at certain levels were less critical for development of

some bacterial diseases than moisture. In this study, combination of high relative humidity and low night temperature caused cumulative effect on the disease caused by PC4, but did not affect disease severity caused by PV3. Similarly, when these factors were combined with fertilization rates they induced higher length of pith necrosis. In the presence of 150 ppm nitrogen and zero potassium a significant increases in disease severity was observed (Table 4 and Fig. 1).

The present results indicate that high nitrogen fertilization, high relative humidity and low relative night temperature favore the development of succulent, soft pith of plants which results in increased disease severity by the pathogens studied. On the other hand, potassium applications provided significant reduction in disease severity of pith necrosis. In the Aegean region of Turkey, high relative humidity and low night temperatures often occur during winters in greenhouses which are not heated. A good control of tomato pith necrosis could be achieved by balancing fertilization of plants and by eliminating the effect of high relative humidity and low night temperatures.

ÖZET

AZOT, POTASYUM, YÜKSEK ORANTILI NEM VE DÜŞÜK GECE SICAKLIĞININ DOMATES ÖZ NEKROZU HASTALIĞINA ETKİSİ

Sera çalışmalarında dört ayrı denemede, saksılarda yetiştirilen 1 aylık domates bitkileri PC4 (*Pseudomonas cichorii*), PV3 (*Pseudomonas viridiflava*) ve EC2 (*Erwinia carotovora* subsp. *carotovora*) izolatlarının 10^8 h/ml süspansyonu ile yapay olarak inokule edilmişler ve nekroz uzunluğuna dayanan değerlendirme, inokulasyondan 1 ay sonra yapılmıştır. 0, 150, 300 ve 450 ppm dozda nitrat ve amonyum olarak uygulanan azot, bitki gelişmesini etkilemiş ve yüksek dozlarda fitotoksiteye neden olmuştur. Nitrat azotunun tüm dozları ve amonyumun 150 ppm dozu PV3 ve EC2 enfeksiyonlarını arttırmış, amonyumun diğer dozlarında ise etki saptanmamıştır. Gerek nitrat, gerekse amonyum azotu PC4 enfeksiyonunu azaltmıştır. Potasyum uygulamaları (0, 200, 400 ve 600 ppm) ve PC4, PV3 ve EC2 izolatlarının oluşturdukları nekroz uzunlukları arasında negatif korelasyon saptanmıştır. Yüksek orantılı nem PC4, PV3 ve EC2 nekroz uzunluklarını sırasıyla %22.17, %23.33, %42.59 arttırmıştır. Düşük gece sıcaklığı PC4 ve EC2 izolatlarının oluşturduğu nekroz uzunluklarını sırasıyla %15.17, %37.44 oranında arttırmış, ancak PV3'ü önemli derecede (P=0.05) etkilememiştir. Yüksek orantılı nem ve düşük gece sıcaklığı koşulları birlikte PC4 ve EC2'nin nekroz uzunluklarını sırasıyla %42.52 ve %42.38 oranında arttırırken, PV3'ü etkilememiştir. Hastalığın en fazla görüldüğü azot dozu, potasyum noksanlığı, yüksek orantılı nem ve düşük gece sıcaklığı faktörlerinin birlikte bulunması PC4, PV3 ve EC2'nin nekroz uzunluklarını sırasıyla %53.89, %54.65, %48.99 oranında arttırmıştır.

Anahtar Kelimeler: Domates öz nekrozu hastalığı, N'lu ve K'lı gübreler, orantılı nem, sıcaklık

ACKNOWLEDGMENTS

We thanks Prof. Dr. H. Çolakoğlu for advice and counsel during the course of the study. We are greateful to Ministry of Agriculture of Turkey that supported the study as a part of Agricultural Research Project (TAP).

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J. Turk. Phytopath., Vol. 31, No. 3, 137-154, 2002 ISSN 0378 - 8024

Effects of Extracts from Some Medicinal Plants and Composts on Pectolytic Enzymes Produced in Liquid Culture and on Onion Seeds by *Aspergillus niger* and *Fusarium oxysporum* f. sp. *cepae*

Nuray ÖZER Mustafa MİRİK Ahmet ÇITIR

Department of Plant Protection, Faculty of Agriculture, Trakya University, 59030 Tekirdağ / TURKEY

ABSTRACT

In order to control black mold disease of onion caused by *Aspergilus niger* V. Tieghem (AN) and onion basal rot caused by *Fusarium oxysporum* Schlecht f. sp. *cepae* (Hanzen) Synder & Hansen (FOC), the aqueous extracts of some medicinal plants like sage (*Salvia officinalis* L.), oregano (*Origanum vulgare* L. subsp. *hirtum* (Link) Ietswaart), balm (*Melissa officinalis* L.), peppermint (*Mentha piperita* L.), garlic (*Allium sativum* L.), the composts of sunflower (*Helianthus annuus* L.) and alfalfa (*Medicago sativa* L.) were tested at the dosages of 10% and 20%. Extracts from garlic and the compost of sunflower were evaluated in liquid medium and on seed for inhibition of pectolytic enzymes and isoenzymes of AN. All extracts were tested for FOC in the same manner, while extract from the compost of alfalfa was applied only to seeds for the evaluation of both pathogens. AN produced only Polygalacturonase (PG) enzyme and isoenzymes, FOC exhibited Pectin Lyase (PNL) and Pectate Lyase (PL) together with PG enzyme and their isoenzymes in untreated liquid medium and seeds with any extract. Most of extracts inhibited these enzymes and their isoenzymatic forms in liquid culture. Extract from alfalfa compost and garlic at the dosage of 10 % reduced PG activity of AN during the seed colonization with the rates of 62.04% and 53.24%, respectively. Both treatments also inhibited synthesis of PG isoenzymes. The extract of garlic (10%) reduced PG and PL activities produced by FOC on seed, with the rates of 81.48% and 71.43%, respectively and it completely inhibited isoenzymatic forms of both enzymes. The extract from sunflower compost (20%) has the highest effect (100%) on PG activity produced by FOC on seed. So no PG isoenzyme bands were exhibited when this extract was applied to seeds which were inoculated with FOC. The treatment with peppermint and oregano at the dosage of 20% were highly effective on only PL activity and isoenzymes produced by this pathogen during seed colonization. None of extracts could be effective on PNL isoenzymes of the same pathogen, although peppermint (20%) and sunflower compost extracts (10%) reduced the activity of this enzyme.

Key words: *Aspergillus niger, Fusarium oxysporum* f. sp. *cepae*, medicinal plant extract, compost extract, pectolytic enzyme

EFFECTS OF EXTRACTS FROM SOME MEDICINAL PLANTS AND COMPOSTS ON PECTOLYTIC ENZYMES PRODUCED IN LIQUID CULTURE AND ON ONION SEEDS BY *ASPERGILLUS NIGER* AND *FUSARIUM OXYSPORUM* F. SP. CEPAE

INTRODUCTION

Onion (*Allium cepa* L.) is an important crop of Tekirdağ province and various other provinces in Turkey. *Aspergillus niger* V. Tieghem (AN) and *Fusarium oxysporum* Schlecht f. sp. *cepae* (Hanzen) Synder & Hansen (FOC) are seed and soil borne pathogens (Hayden and Maude, 1992; Hayden et al., 1994; Köycü and Özer, 1997) which were determined as the pathogens associated with seeds and seed parts such seed coat, endosperm and embryo (Köycü and Özer, 1997). These species of fungi caused pre- and post-emergence damping-off and rot on onion sets and bulbs (Özer and Köycü, 1997). The polygalacturonase (PG) enzyme and isoenzymes produced by AN play an important role in colonization of onion seed during development of disease (Özer et al., 1999). FOC also has the ability to produce pectolytic enzymes (Holz and Knox-Davies, 1985). Inhibitors of cell wall degrading enzymes have been implicated in plant disease resistance (Anderson and Albersheim, 1972; Magro et al., 1983; Bugbee, 1993; Favaron et al., 1993 and 1997). Cell walls of dicotyledonous plants contain polygalacturonase-inhibiting proteins (PGIP) having similar physico-chemical properties (Hoffman and Turner, 1984; Cervone et al., 1987 and 1993).

Extracts from plants and composts contain many antifungal compounds, which may be suitable components in integrated plant protection. The range of their effects is rather broad including toxic, plant health enhancing and other effects (Fawcet and Spencer, 1970; Hoitink, 1980; Schmitt, 1994). Inhibitory effect of extracts from different medicinal plants and composts on AN and FO isolated from onion or various other cultured plants was demonstrated against on colony growth (Vir and Sharma, 1986; Assadi and Behrozin, 1988; Filippi, 1989; Naidu, 1990; Oh et al., 1995; Arya et al., 1996; Mohamed et al., 1996; Qasem, 1996; Sobti et al., 1996; Yonucu, 1997; Awad et al., 1998; Rızkı et al., 1998; Shivpuri et al., 1998; Kapoor, 1999; Kaçar, 2000).

The objective of this study was to determine the potential of extracts from some medicinal plants and compost in suppressing the activity of pectolytic enzymes and isoenzymes produced by AN and FOC in culture and during seed germination.

MATERIALS and METHODS

Plant source

Sage (*Salvia officinalis* L.) and balm (*Melissa officinalis* L.) were collected from the experimental plots of Tekirdağ Faculty of Agriculture, Trakya University (Tekirdağ/ Turkey), oregano (*Origanum vulgare* L. subsp. *hirtum* (L.) Ietswaart) from natural flora of Tekirdağ/Turkey and peppermint (*Mentha piperita* L.) was obtained from the experimental plots of Faculty of Agriculture, Çukurova University (Adana/Turkey). All the plants were collected during the spring and summer and their leaves and shoots were air-dried so were stored in fabric bags at room temperature. Garlic (*Allium sativum* L.) bulbs were obtained from local market (Tekirdağ/Turkey) and their cloves were separated and dried in room temperature.

The stalks of sunflower (*Helianthus annuus* L.) and alfalfa (*Medicago sativa* L.) were used as compost materials. They were collected from the experimental plots of Tekirdağ Faculty of Agriculture, Trakya University (Tekirdağ/Turkey) and were air dried at room temperature.

Preparation of extracts from medicinal plants and composts

The stocks of plant extracts were obtained by grinding shoots and cloves (only for garlic) in distilled water (1:1 w/v) separately in a homogenizer for 10 min. The stalks of alfalfa and sunflower were placed in containers separately for the preparation of compost. Tap water was added to adjust the moisture of 100% on a dry weight basis and both containers were turned upside down in every two weeks which were kept at 20±2 °C for four months. After this period of time, drained liquids, which were accumulated at the bottom of the containers were used as stock solution of composts (Kaçar, 2000).

The stock solutions obtained from the plants and composted materials were filtered through cheesecloth and centrifuged at 3000 rpm for 5 min. The supernatant were filtered aseptically through membrane (Millipore) of pore size, 0.45 µm. They were used at 10% and 20% (v/v) dosages in the experiments. All extracts were applied to FOC isolates, while garlic and plant compost extracts were applied only to AN since inhibitory effects on colony growth of the fungi were effective at these dosages as observed earlier (Kaçar 2000).

Fungal cultures and extract treatment

One isolate (An6) of AN and FOC (Fo6) obtained from naturally infested onion seeds and sets were used in the experiments (Köycü and Özer, 1997). Both isolates were the most virulent to onion if applied to seed (Özer and Köycü, 1997). Fungal cultures were maintained on potato dextrose agar (PDA) (Oxoid). AN was incubated at 30 °C, for 5 days and FOC at 25 °C for 7 days in all tests.

Czapek's liquid medium (pH 4.0) containing 1% (w/v) citrus pectin (Sigma) as sole carbon source was used as the medium. Extracts were incorporated and mixed well with this medium at 10% and 20% (v/v) dosages separately. Agar discs (6 mm in diameter) were cut from the edge of cultures on PDA. The cultures were grown in 250 ml Erlenmayer flasks containing 50 ml liquid medium. The extract from alfalfa compost however was not treated to liquid medium, which has revealed solidification effect in liquid medium.

Seed inoculation and extract treatment

Onion seeds of the cultivar Kantartopu, which is susceptible to both pathogens (Özer, 1998), were used in all experiments. The seeds were surface-sterilized in a solution of sodium hypochlorite (1%) for 5 min, rinsed for 5 min in sterile distilled water, then

finally air dried on sterile filter paper. The sterilized seeds were treated with the extracts by mixing and shaking the two for 30 min. They were then inoculated with AN by employing the procedure of Hayden et al. (1994). For the inoculation of FOC the procedure was utilized as described by Özer and Köycü (1997) by applying a suspension of $1X10^6$ spores/ml. In the control, no extract was applied to inoculated seeds. Sixteen replicates, each containing 25 seeds per treatment were placed on sterile filter paper (Blotter method) moistened with sterilized distilled water in 9 cm petri dishes as they were incubated in the dark for seven days.

Enzyme extraction from fungal cultures and onion seeds

Three days-old cultures of AN and five days-old cultures of FOC were collected from liquid media and the mycelium was removed by filtration using a Duran funnel.

The enzyme preparations from the seeds treated with extracts and isolates were obtained by grinding infected tissues in an ice-cooled mortar in 0.05M Tris-HCL buffer pH 7.8 (1 ml/g tissue) containing 0.1M KCL, 0.5% (w/v) cysteine and 1% (w/v) insoluble polyvinylpolypyrrolidone (Sigma). The slurry was then strained through three layers of cheesecloth. Filtrates from liquid culture and seeds were centrifuged at 15.000 g for 20 min at 4 °C. The supernatant was filtered through the filtre (Millipore) of pore size, 0.22 µm and dialysed against several changes of distilled water at 4 °C. The same procedures were applied to the untreated liquid culture and seeds.

Pectolytic enzymes assays

PG activity was determined by measuring the increase of reducing-end groups over time by modified the method of Nelson (1944), using D-galacturonic acid (GA) (Sigma) as a standard. One unit of PG activity (RU) produced 1 µmol of reducing group/min from 0.25% (w/v) polygalacturonic acid (PGA) (Sigma) in Na-acetate buffer (0.1M, pH 4.0) at 35 °C. PNL (pectin lyase) and PL (pectate lyase) activity was assayed spectrophotometrically by measuring the increase of absorbance at 235 nm. An increase of 1.73 indicated the formation of 1 µmol of unsaturated uronide (Zucker and Hankin, 1970). One unit of PNL activity (PNLU) catalyzed the formation of 1 µmol of unsaturated uronide/min from 0.25% (w/v) citrus pectin in Tris HCL buffer (0.1 M, pH 8.0) at 35 °C. One unit of PL activity (PLU) catalyzed the same amount of formation from 0.25% (w/v) PGA in the same conditions. All the assays were repeated three times.

Isoenzyme identification

Isoenzyme separation by isoelectric focusing (IEF) was realised horizontally on Mini IEF cell apparatus (Bio-Rad) by using 0.4 mm thick polyacrylamide gels containing 5% (v/v) ampholyte (Sigma) covering the pH range of 3.5 to 10.0. The gels were run at 200 V, 450 V, 600 V and 950 V for 15, 30, 20 and 25 minutes, respectively.

The gels were overlaid with ultrathin (0.4 mm) agarose gels after IEF, prepared as described by Ried and Collmer (1985). In order to detect PG isoenzyme, 1% (w/v) agarose (Sigma) gel contained 0.1% (w/v) PGA buffered at pH 4.0 with 50 mM Na-acetate. The 1% (w/v) agarose gel for PNL and PL detection contained 0.1% (w/v) pectin and PGA respectively in 50 mM Tris-HCL buffer, pH 8.0. IEF polyacrylamide gels overlaid with ultrathin agarose gel were incubated at 100% humidity from 30 to 60 min at 35 °C. Activity bands were visualised by staining the agarose overlay for 30 min in 0.05% (w/v) ruthenium red (Sigma), followed by rinsing in distilled water.

Data analysis

The isoelectric point (pI) values of pectolytic isoenzymes were estimated from a regression equation of standard protein versus the distance migrated. Data on enzyme activity was analyzed using the analysis of variance and means separated according to Duncan Multiple Range Test (P=0.05).

RESULTS and DISCUSSION

Pectolytic enzymes of AN in liquid culture and on onion seeds after treatments

AN produced only PG in liquid culture. PG activity of AN in liquid culture was reduced significantly in the treatment of garlic extract at both dosages in comparison with the treatments of sunflower compost and control as indicated in Table 1. Garlic extract at the dosage of 10% however was the most effective (100%) on this enzyme activity. This treatment also reduced PG activity of fungus on inoculated seeds giving the effect of 53.24%. On the other hand, the lowest enzyme activity was observed on seeds when they treated with alfalfa compost extract at the dosage of 10%. Sunflower compost treatment was not effective on PG activity of AN in liquid culture and on seed.

Isoenzymes of AN in liquid culture and on onion seeds after treatments

The dialysed culture and infected onion seed filtrates were subjected to thin-layer polyacrylamide gel IEF and PG isoenzymes were evaluated. PNL and PL isoenzymes were not detected in any culture filtrates or in seeds. Six acidic PG isoenzymes at pI 5.8, 5.4, 5.2, 4.7, 4.5 and <3.5 were found on untreated culture filtrates and colonized seeds. Any isoenzyme production was not detected in the liquid culture treated with garlic extracts at the dosage of 10% (Fig. 1A). Isoenzyme band at pI <3.5, produced by the pathogen on untreated seed, was inhibited by all extracts which applied to the seeds (Fig. 1B). The bands at pI 5.4, 5.2 and 4.7 however were resolved as faint bands after the seed treatments with extracts from garlic and sunflower compost at both dosages. All PG bands of pathogen appeared much less intense after the treatment of seeds with alfalfa compost extract at both dosages, compared with the other treatments.

Table 1. Effects of different extracts on PG activity of AN in liquid culture and on seed

Extract studied	Dosage (%)	PG activity (RU)[a] Liquid culture	Effect of extract (%)	Treated seed	Effect of extract (%)
Garlic	10	0.00 b[b]	100.00	1.01 b	53.24
	20	0.24 b	77.14	1.44 ab	33.33
Sunflower	10	0.80 a	23.80	2.41 a	-
compost	20	0.90 a	14.28	2.31 a	-
Alfalfa	10	ND	-	0.82 b	62.04
compost	20	ND	-	0.94 b	56.48
Control	-	1.05 a	-	2.16 a	-

ND: no data.

[a] Values are expressed as means of three replicates

[b] Means within a column, followed by the same letters are not significantly different (P< 0.05) according to Duncan 's multiple range test.

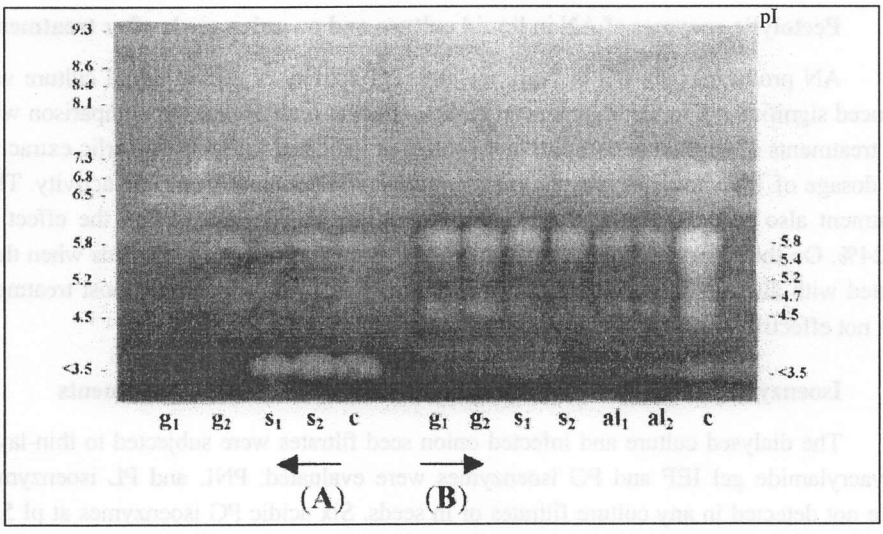

Figure 1. PG isoenzymes of AN from liquid culture (A) and on onion seed (B) treated with different extracts. g: garlic; s: sunflower compost; al: alfalfa compost; c: control. 1: dosage of 10%; 2: dosage of 20%. Position of PG bands is indicated on the right. Position and pI values of IEF markers are shown on the left.

Pectolytic enzymes and isoenzymes of FOC in liquid culture after treatments

PG, PNL and PL activities of FOC isolate in liquid culture and the effects of extracts on these enzymes are presented in Table 2. Most of the extracts added in the two

different dosages to liquid culture reduced its pectolytic enzyme activity. However, the garlic extracts at the dosage of 10% and 20% were highly effective on PG, PNL and PL activity of the pathogen. The oregano (10%), peppermint (20%) and sunflower (10% and 20%) treatments followed it. Peppermint extract, at the dosage of 10%, increased PG activity of the pathogen, but this induction was not significant. Nevertheless this extract was detected as highly effective on PNL (100%) and PL (98.74%) activities.

Table 2. Effects of different extracts on PG, PNL and PL activity of FOC in liquid culture

Extract studied	Dosage (%)	PG activity (RU)[a]	Effect of extract (%)	PNL activity (PNLU)[a]	Effect of extract (%)	PL activity (PLU)[a]	Effect of extract (%)
Sage	10	0.27 cd[b]	63.01	0.56 ab	53.33	0.11 b	95.40
	20	0.37 bc	49.31	0.08 b	93.33	0.17 b	92.89
Oregano	10	0.09 cd	87.67	0.08 b	93.33	0.00 b	100.00
	20	0.39 bc	46.57	0.00 b	100.00	0.00 b	100.00
Balm	10	0.20 bd	72.60	0.28 ab	76.67	0.33 b	86.19
	20	0.65 ab	10.96	0.83 ab	30.83	0.61 b	74.48
Peppermint	10	0.78 a	-	0.00 b	100.00	0.03 b	98.74
	20	0.21 cd	71.23	0.00 b	100.00	0.05 b	97.91
Garlic	10	0.00 d	100.00	0.05 b	95.83	0.02 b	99.16
	20	0.00 d	100.00	0.01 b	99.17	0.01 b	99.58
Sunflower	10	0.03 cd	95.89	0.05 b	95.83	0.23 b	90.38
compost	20	0.13 cd	82.19	0.36 ab	70.00	0.31 b	87.03
Control	-	0.73 a	-	1.20 a	-	2.39 a	-

[a] Values are expressed as means of three replicates.

[b] Means within a column, followed by the same letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

As indicated in Fig.2, six PG bands at pI 8.9, 8.6, 6.1, pI 5.8, 5.4 and 5.2 were detected from untreated liquid cultures. Most of the extracts inhibited the production of the bands. However, one PG isoenzymatic band (pI 8.9) of pathogen was appeared after the treatments with the extracts from balm, peppermint and sunflower compost, at the dosages of 10%, 10% and 20%, respectively.

Two PNL isoenzymatic bands at pI 8.9 and 8.4 were present in the untreated filtrates (Fig. 3). The extracts from oregano (20%), balm (20%), peppermint (20%), garlic (10% and 20%) and sunflower compost (10%) completely inhibited the isolate to develop PNL isoenzymes in liquid culture. The extracts of sage (20%) and oregano (10%) however inhibited the exhibition of PNL isoenzymatic band at pI 8.9. When sunflower compost extract was used at the dosage of 20%, only this isoenzymatic band was exhibited. As a result of the other treatments, the pathogen produced PNL isoenzymes as in control. Isoenzymatic band at pI 7.5 was also appeared in the treatments of sage at both dosages.

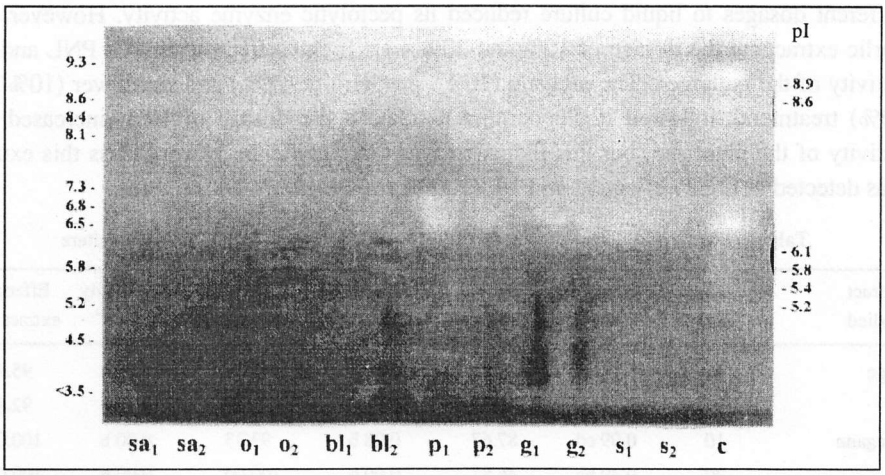

Figure 2. PG isoenzymes of of FOC from liquid culture treated with different extracts. sa: sage; o: oregano; bl: balm; p: peppermint; g: garlic; s: sunflower compost; c: control; 1, dosage of 10%; 2: dosage of 20%. Position of PG bands is indicated on the right. Position and pI values of IEF markers are shown on the left.

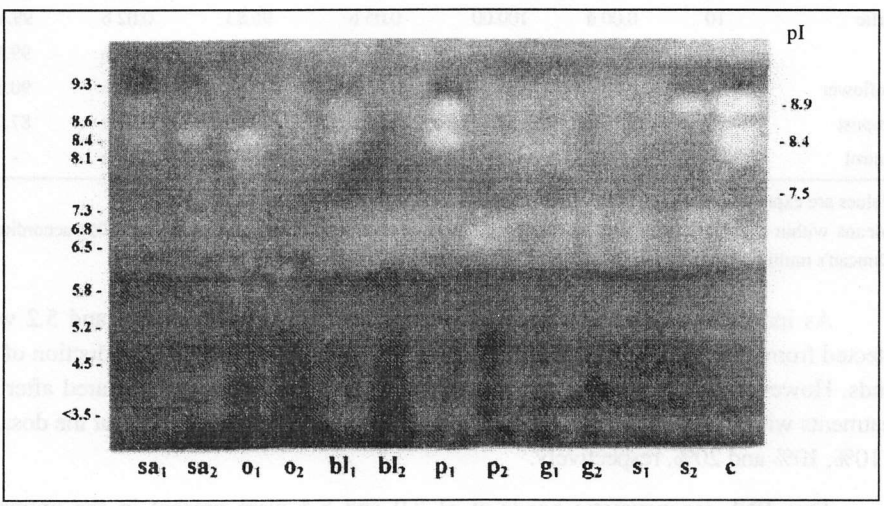

Figure 3. PNL isoenzymes of FOC from liquid culture treated with different extracts. sa: sage; o: oregano; bl: balm; p: peppermint; g: garlic; s: sunflower compost; c: control. 1, dosage of 10%; 2: dosage of 20%. Position of PNL bands is indicated on the right. Position and pI values of IEF markers are shown on the left.

Two PL isoenzymatic bands at pI 8.9 and 8.4 were exhibited in the untreated filtrates of the pathogen. As revealed in Fig.4, same extracts were effective for the inhibition of PL isoenzymes as in PNL isoenzymes. However no isoenzymatic bands were exhibited at the

treatments of sage and oregano extracts at the dosages of 20% and 10% respectively. Sage treatment at the dosage of 10% however induced FOC for the production of PL isoenzymes. When this extracts was used in liquid culture, the bands at pI 8.1 and 7.5 also appeared.

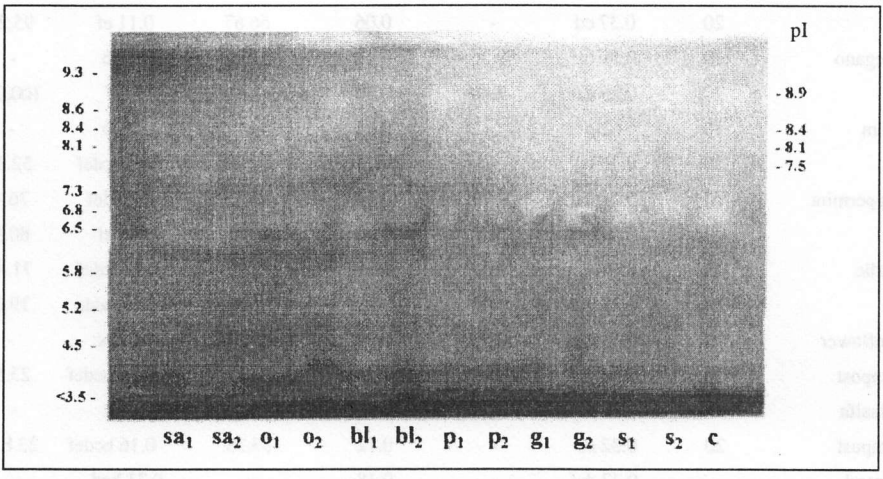

Figure 4. PL isoenzymes of FOC from liquid culture treated with different extracts. sa: sage; o: oregano; bl: balm; p: peppermint; g: garlic; s: sunflower compost; c: control. 1, dosage of 10%; 2: dosage of 20%. Position of PL bands is indicated on the right. Position and pI values of IEF markers are shown on the left.

Pectolytic enzymes and isoenzymes of FOC on inoculated seeds after treatments

As shown in Table 3, PG activity was significantly decreased with the treatments of garlic (10%) and sunflower compost (20%). The extract of sunflower compost, at the dosage of 10%, was the most effective (83.33%) treatment for the inhibition of PNL activity. Peppermint treatment (20%) followed it. However the differences in PNL activities were not significant as compared with control. The extract of sage (20%), oregano (20%) and peppermint (20%) significantly reduced PL activity, showing the effects of 95.24%, 100% and 80.95%, respectively. Peppermint (10%) and garlic (10%) treatments followed them.

Extracts from sage and balm at both dosages, peppermint at the dosage of 20% increased PG activity. Alfalfa compost extract (10%) induced the activity of PG, PNL and PL, but this induction was significant only for PL activity.

As revealed in Fig. 5, one PG isoenzyme band was detected at pI 9.3 from the untreated seeds. Extracts of balm and peppermint at the dosage of 10% also induced the production of isoenzymatic band at pI 8.9 and 9.3. Sunflower compost treatment at the dosage of 10% induced the production of acidic isoenzyme bands at pI 6.1 and 5.8,

Table 3. Effects of different extracts on PG, PNL and PL activity of FOC on seed

Extract studied	Dosage (%)	PG activity (RU)[a]	Effect of extract (%)	PNL activity (PNLU)[a]	Effect of extract (%)	PL activity (PLU)[a]	Effect of extract (%)
Sage	10	0.53 ab[b]	-	0.16	11.11	0.07 cdef	66.67
	20	0.37 cd	-	0.06	66.67	0.11 ef	95.24
Oregano	10	0.11 fg	59.26	0.13	27.78	0.32 b	-
	20	0.25 def	7.40	0.20	-	0.00 f	100.00
Balm	10	0.62 a	-	0.74	-	0.31 b	-
	20	0.46 bc	-	0.14	22.22	0.10 cdef	52.38
Peppermint	10	0.21 def	22.22	0.14	22.22	0.05 def	76.19
	20	0.31 cde	-	0.04	77.78	0.04 ef	80.95
Garlic	10	0.05 g	81.48	0.16	11.11	0.06 cdef	71.43
	20	0.15 efg	44.44	0.14	22.22	0.17 bcde	19.05
Sunflower	10	0.11 fg	59.26	0.03	83.33	0.22 bc	-
compost	20	0.00 g	100.00	0.21	-	0.16 bcdef	23.81
Alfaalfa	10	0.30 de	-	0.24	-	0.63 a	-
compost	20	0.32 cd	-	0.12	33.33	0.16 bcdef	23.81
Control	-	0.27 def	-	0.18	-	0.21 bcd	-

[a] Values are expressed as means of three replicates.

[b] Means within a column, followed by the same letters are not significantly different ($P< 0.05$) according to Duncan's multiple range test.

whereas these bands were not exhibited by the pathogen on untreated seeds. The pathogen could not produce PG isoenzymes in rest of the other treatments.

PNL isoenzyme bands at pI 9.3, 8.9, 5.8, 5.4, 4.7, 4.2 were obtained from untreated seeds (Fig. 6). None of the extracts was effective on PNL isoenzymes of the pathogen during seed colonization. Only alfalfa compost extract inhibited the production of alkaline bands as this treatment and oregano extract induced the development of acidic isoenzymes. The isoenzymatic band at pI 8.6 was appeared after the treatments of sage, balm, peppermint and compost of sunflower as sage treatments also induced PNL isoenzymatic band at pI 8.1.

Two basic PL isoenzymatic bands (pI 8.9 and 8.6) were appeared from seeds treated with extracts of sage, balm and sunflower compost at the both dosages as shown in Fig. 7 like untreated seeds. FOC produced acidic bands at pI 6.1, pI 5.8, pI 5.2 on the seeds treated with alfalfa compost extract at the dosage of 10%, although these bands were not detected in the enzyme extracts from untreated seeds. Extracts of oregano, peppermint and garlic inhibited the production of PL isoenzymes of this pathogen.

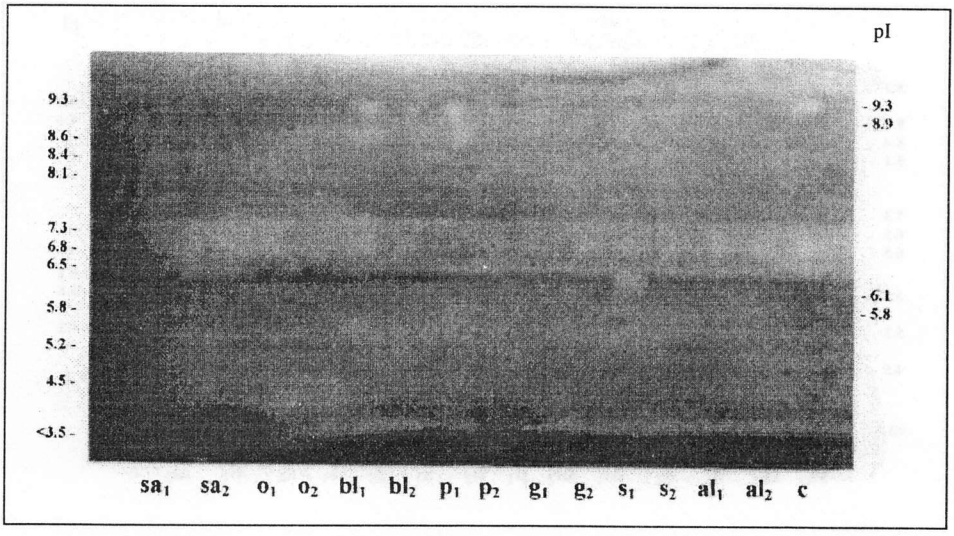

Figure 5. PG isoenzymes of FOC on seed treated with different extracts. sa: sage; o: oregano; bl: balm; p: peppermint; g: garlic; s: sunflower compost; al: alfalfa compost; c: control. 1, dosage of 10%; 2: dosage of 20%. Position of PG bands is indicated on the right. Position and pI values of IEF markers are shown on the left

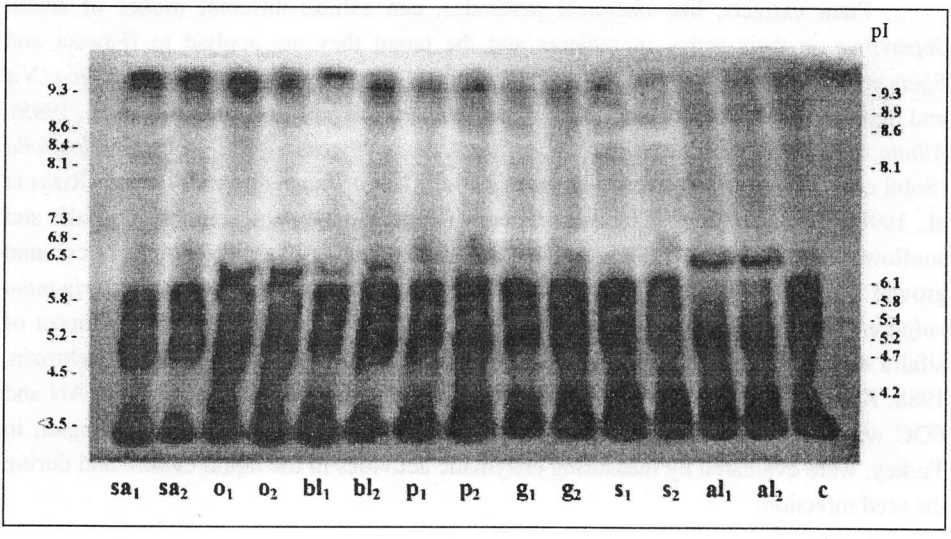

Figure 6. PNL isoenzymes of FOC on seed treated with different extracts. sa: sage; o: oregano; bl: balm; p: peppermint; g: garlic; s: sunflower compost; al: alfalfa compost; c: control. 1, dosage of 10%; 2: dosage of 20%. Position of PNL bands is indicated on the right. Position and pI values of IEF markers are shown on the left.

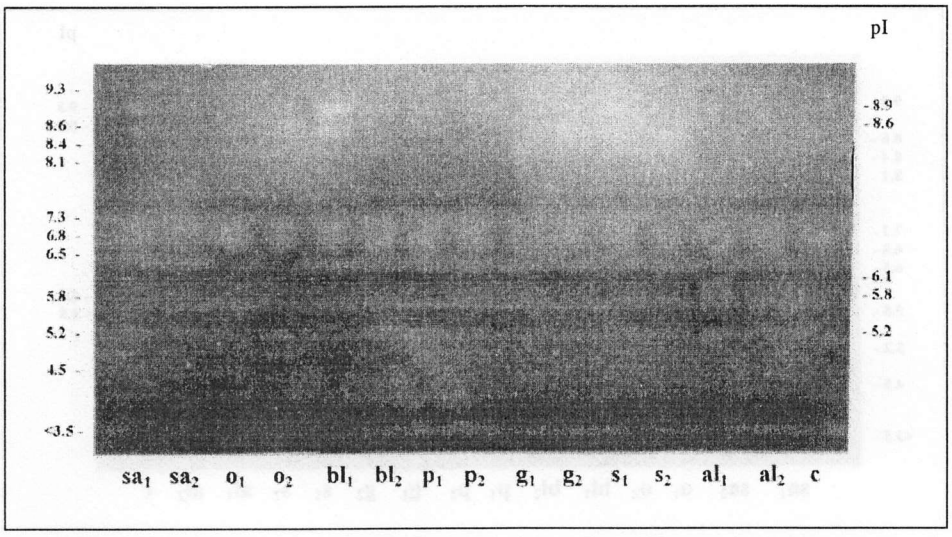

Figure 7. PL isoenzymes of FOC on seed treated with different extracts. sa: sage; o: oregano; bl: balm; p: peppermint; g: garlic; s: sunflower compost; al: alfalfa compost; c: control. 1: dosage of 10%; 2: dosage of 20%. Position of PL bands is indicated on the right. Position and pI values of IEF markers are shown on the left.

Plant extracts, like chemical pesticides, can exhibit different modes of action depending on their active ingradients and the target they are applied to (Fawcet and Spencer, 1970; Schmitt, 1994). In previous studies, extracts from *Azadirachta indica* (Vir and Sharma, 1986; Sobti et al., 1996), *Coptis chinensis*, *Rheum polatum* (Oh et al., 1995), *Allium sativum* (Arya et al., 1996; Kaçar, 2000), *Ocimum gratissimum, Polyalthia logifolia* (Sobti et al., 1996), *Piper bettle* (Mohamed et al., 1996), *Trachyspermum ammi* (Rızkı et al., 1998), *Curcuma longa, Zingiber officinale* (Kapoor, 1999), the compost of alfalfa and sunflower stalks (Kaçar, 2000) have been determined having inhibitory effects of colonial growth of AN. It was also proved that the extracts from *Salvia officinalis Origanum vulgare subsp. hirtum, Mentha piperita, Melissa officinalis, Allium sativum,* compost of alfalfa and sunflower stalks inhibited the colony growth of FOC (Assadi and Behrozin, 1988; Kaçar, 2000). For the first time in this study the effect of plant extracts on AN and FOC which are important pathogens of diseases on onion grown in Trakya Region in Turkey, were evaluated by measuring enzymatic activities in the liquid culture and during the seed infection.

The pathogens usually produce a set of pectolytic enzymes, which are supposed to be an important virulence factors (Bateman and Basham, 1976; Cooper, 1984; Collmer and Keen, 1986; Alghisi and Favaron, 1995). Özer et al. (1999) reported that AN produced PG enzyme and isoenzymes in liquid culture and on onion seeds as well as it

was able to produce that enzyme and isoenzymes in the filtrates from untreated liquid culture and seeds. FOC produced PG, PNL and PL and their isoenzymes in the similar manner to AN. It was also determined that FOC produced an exo-PG and endo-PTE (endo-pectin-transeliminase) *in vitro* or in the cell walls of onion bulbs (Holz and Knox-Davies, 1985).

Extracts used in this study had different effects on pectolytic enzymes and isoenzymes which produced by AN and FOC in liquid culture or on inoculated seeds. Most of them were effective on pectolytic enzymes and isoenzymes in liquid culture. Garlic extract at the dosage of 10% reduced the PG activity and isoenzymes significantly which were produced by AN in both conditions. Alfalfa compost extract however used only in seed treatment was more effective on PG activity and its isoenzymatic forms of AN than garlic extract. Garlic extract at 10% dosage also inhibit PG and PL activities and their isoenzymes which produced by FOC during seed colonization. Sunflower compost extract at the dosage of 20% was completely inhibited the PG activity and its isoforms which produced by this fungus species on seed. In the same manner, oregano and peppermint extracts at the dosage of 20% reduced PL activity and isoenzymes of this pathogen on seed. Most of the studies have been reported on this case were related to protein inhibitors of cell wall degrading enzymes produced by pathogens (Cervone et al., 1993; Favaron et al., 1993). The extract obtained from the roots of sugar beet was found effective against PNL from *Rhizoctonia solani* and *Phoma betae* (Bugbee, 1993). Extracts of onion and leek contain proteins (PGIP) that inhibit polygalacturonases (PGs) produced in vitro by *Sclerotinia sclerotiorum, Botrytis cinerea, Fusarium moniliforme, Phoma terrestris, Sclerotium cepivorum, Macrophomina phaseolina, Didymella bryoniae* and *Phoma lycopersici* (Favaron et al., 1993). Favaron et al. (1997) found a close relationship between PGIP activity *in vitro* and the ability of PGIP to protect leek tissue from endo-PG degradation of *F. moniliforme, S. sclerotiorum, B. aclada* and *S. cepivorum*. Extracts from garlic, compost of sunflower and alfalfa seem to be alternative inhibitors of PG and its isoenzymes produced by AN and FOC or both in the present study. Beside this fact the extracts of oregano (20%), peppermint (20%) and garlic (10%) probably contained the factors inhibiting PL enzyme and isoenzymes of FOC. So far none of extract treatments, which were tested in this study as treatments effectively controlled the PNL isoenzymes of this pathogen.

Pectolytic enzymes and their isoenzymes may have different functions and they may be produced at different stages of plant (Keon et al., 1987). Plant extracts and composts determined as effective in this study should be examined in the potted plants and field trial experiments for their inhibition of pectolytic enzyme activity produced by AN and FOC during the seedling and set development.

EFFECTS OF EXTRACTS FROM SOME MEDICINAL PLANTS AND COMPOSTS ON PECTOLYTIC ENZYMES PRODUCED IN LIQUID CULTURE AND ON ONION SEEDS BY *ASPERGILLUS NIGER* AND *FUSARIUM OXYSPORUM* F. SP. CEPAE

ÖZET

BAZI TIBBİ BİTKİLER VE KOMPOST EKSTRAKTLARININ *Aspergillus niger* ve *Fusarium oxysporum* f. sp. *cepae* TARAFINDAN SIVI KÜLTÜRDE VE SOĞAN TOHUMLARI ÜZERİNDE ÜRETİLEN PEKTOLİTİK ENZİMLER ÜZERİNE ETKİSİ

Bu çalışmada bazı tibbi bitkilerin [adaçayı (*Salvia officinalis* L.), İstanbul kekiği (*Origanum vulgare* L. subsp. *hirtum* (Link) Ietswaart), melisaotu (*Melissa officinalis* L.), bahçe nanesi (*Mentha piperita* L.) ve sarmısak (*Allium sativum* L.)], ayçiçeği (*Helianthus annuus* L.) ve yonca (*Medicago sativa* L.) sapı kompostlarının iki farklı dozda (%10 ve %20) su ile hazırlanan ekstraktları, soğanda (*Allium cepa* L.) patojen olan *Aspergillus niger* V. Tieghem (AN) ve *Fusarium oxysporum* Schlecht f. sp. *cepae* (Hanzen) Synder&Hansen (FOC) izolatları kullanılmıştır. Ayçiçeği kompostu ve sarmısaktan elde edilen ekstraktlar sıvı kültüre ve soğan tohumlarına uygulanmış, AN'nin sıvı kültürde ve soğan tohumları üzerinde ürettiği pektolitik enzim ve izoenzimleri üzerine etkileri belirlenmiştir. Bununla beraber ekstraktların tümü FOC için aynı şekilde test edilmiş, yonca kompostu ekstraktları ise sadece tohumlara uygulanarak her iki fungus için kullanılmıştır. Herhangi bir ekstrakt ile uygulama yapılmamış sıvı kültür ve tohumda, AN sadece poligalakturonaz (PG) enzim ve isoenzimlerini, FOC ise PG, Pectin Liyaz (PNL) ve Pektat Liyaz (PL) enzim ve izoenzimlerini üretmiştir. Test edilen ekstraktların çoğu sıvı kültürde bu enzim ve izoenzimlerin üretimini engelllemiştir. %10 dozundaki yonca kompostu ve sarmısak ekstraktları AN 'nin tohum kolonizasyonu süresince PG aktivitesini sırasıyla %62.04 ve %53.24 etki göstererek azaltmıştır. Bu ekstraktlar aynı zamanda patojenin PG izoenzimlerinin sergilenmesini de engellemiştir. Sarmısak ekstraktı (%10), FOC tarafından tohum üzerinde üretilen PG ve PL aktivitelerini sırasıyla %81.48 ve %71.43 etki göstererek azaltmış, söz konusu enzimlerin izoenzimatik formlarının oluşumunu tamamen engellemiştir. Yine ayçiçeği kompostu ekstraktı (%20) tohumlara uygulandığında FOC tarafından tohumda üretilen PG aktivitesi üzerine en yüksek oranda (%100) etkili olmuş ve herhangi bir PG izoenzim bandı sergilenememiştir. %20 dozundaki nane ve kekik ekstraktları aynı patojenin tohum kolonizasyonu süresince ürettiği enzimlerden sadece PL aktivitesi ve izoenzimleri üzerine yüksek derecede etkili olmuşlardır. Nane (%20) ve ayçiçeği kompostu (%10) ekstraktları FOC'un PNL aktivitesini azaltsa da, ekstraktların hiçbiri PNL izoenzimleri üzerine etkili olamamıştır.

Anahtar Kelimeler: *Aspergillus niger, Fusarium oxysporum* f. sp. *cepae*, tıbbi bitki ekstraktı, kompost ekstraktı, pektolitik enzim

ACKNOWLEDGMENTS

Research supported by Turkish Scientific and Technical Research Council (TÜBİTAK) (TARP-2072) and the Research Fund of Trakya University (TÜAF-186). The authors wish to thank Prof. Paolo Magro (Italy) and Dr. Gabriele Chilosi (Italy) for their scientific assistance.

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J. Turk. Phytopath., Vol. 31, No. 3, 155-165, 2002 ISSN 0378 - 8024

Possible Correlation of Grapevine Virus D to Rugose Wood Disease of Grapevine

N. BUZKAN* **A. MINAFRA**** **G. P. MARTELLI*****

* Department of Plant Protection, Faculty of Agriculture University of Sutcu Imam, 46060 Kahramanmaras / TURKEY

** Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari / ITALY

*** Dipartimento di Protezione delle Piante dalle Malattie e Microbiologia Applicata, Università degli Studi di Bari, 70126 Bari / ITALY

ABSTRACT

An apparently new sap-transmissibile *Vitivirus, Grapevine virus D* (GVD), was isolated in Apulia (Southern Italy) from a grapevine with corky rugose wood symptoms. Virus specific-DNA primers were designed based on the nucleotide sequence of the 3'terminal region of the viral genome. The size of amplified DNA products was 700 bp. No-amplification was obtained when the primers were used for GVA and GVB extracts from infected grapevine and herbaceous plants. An investigation for the presence of GVD, carried out with RT-PCR on 307 vines of diverse varieties and geographical origins, showed that the virus occured in ca. 4% of 218 accessions with rugose wood symptoms, but in none of 89 disease-free vines. Viral particles, filamentous with distinct cross-banding and size of 825 nm, were decorated with the virus-specific antisera, but did not react to GVA, GVB and GVC particles. A digoxygenin-labeled DNA (DIG-DNA) probe was used to confirm the specificity of GVD DNA bands by hybridizing PCR products in Southern blot.

Key words: Grapevine, Rugose wood disease, Grapevine virus, GVD

INTRODUCTION

Grapevine virus D (GVD) is little known and taxonomically unassigned new virus. It was isolated in Apulia (Southern Italy) by mechanical transmission to *Nicotiana occidentalis* from *in vitro*-grown vine of *Vitis vinifera* cv. Primus affected by "rugose wood". No serological relationship was detected by ELISA with tricho- and closteroviruses (Bonavia et al., 1996). A virus-specific antiserum, produced at the University of Bari. Viral particles are filamentous with distinct cross-banding and size of 825x12 nm. Coat protein subunits have an estimated weight of c. 7600 nt in size. The patterns obtained by dsRNA analysis suggest that GVD is a tentative species in the *Trichovirus* genus (Abou-Ghanem et al., 1997). The virus was later classified in the *Vitivirus* genus due to its particle structure, biological and physico-chemical properties (Martelli et al., 1997).

The availability of nucleotide sequence cloned from 3' end region of RNA genome gave an idea to design DNA primers specific for standard reverse transcription polymerase chain reaction (RT-PCR) in infected grapevine tissue in order to define the incidence of virus infection in the plants and correlate it with a possible disease.

MATERIALS and METHODS

Collection of plant samples: Mature cuttings from different grapevine varieties in the collection of the University of Bari at Valenzano and from *V. vinifera* cv. Primus at Castellaneta districts in Italy. Samples were also collected from the registered clones that had been submitted to a sanitation and are of certified category (Table 1).

Table 1. Grapevine samples collected for GVD detection with RT-PCR

Grapevine samples	Number of samples
Vines with rugose wood	168
Vines without rugose wood	75
V. vinifera cv. Primus	50
Registered vines	14

Primer selection. The primers were designed by computer analysis (Primer Detective, Ver. 1.01, Clontech. Labs. Inc.) of nucleotide sequence of GVD pGD8dt (Abou-Ghanem et al., 1997) (Table 2). These primers were assured absence of homology at the 3' and 5' end of each primer set pair (Lowe et al., 1990). Primers were synthesized by GENENCO-Life Science.

Table 2. GVD DNA primers for PCR amplification

Primer	Bases	Sequence	DNA product (bp)
C395	22	5'GTACCTTAGGACGCTCTTCGGG3'	700
H122	22	5'GCAACCCAGCTCACACTCATGC3'	

RT-PCR based on plant extracts and total nucleic acids: Plant crude extracts were obtained according to Wetzel et al., (1991), as described by Minafra and Hadidi (1994). About 100 mg of phloem scrapings from dormant cuttings were ground with the presence of extraction buffer [50 mM sodium citrate, pH 8.3, 20 mM diethyldithiocarbamate (DIECA), 2% polyvinylpyrrolidone (PVP)]. Extracts were clarified at 8.000 rpm for 10 min for further processing.

Total nucleic acids (TNAs) were obtained from both grapevine (phloem scrapings and leaf petioles) and herbaceous host tissues (*Nicotiana clevelandii*) as described by White and Kaper (1989) with slight modifications. Around 100 mg of herbaceous host

tissues were powdered in a mortar and pestle with liquid nitrogen until the tissue became light green. Powdered tissue was extracted with 3 ml of 0.1 M glycine-NaOH, pH9.0, containing 50 mM NaCl, 10 mM EDTA, 2% SDS and 1% sodium lauroylsarcosine and equal volume of buffer-saturated phenol and chloroform. Nucleic acid precipitation was made with 2.5 vol of cold absolute ethanol and 0.1 vol of 3 M sodium acetate, pH 5.5. The precipitate was resuspended in sterile-distilled water. TNAs extracted from grapevine were further purified from residual unwanted plant components with CF11. After phenol: chloroform treatment, aliquots of 1 ml were treated with 50 mg of CF11 (Whatman, Maidstone, England). Absolute ethanol was added to final concentration to favour total nucleic acids binding to cellulose. Nucleic acids were eluted from cellulose in presence of 400 µl of 1X STE buffer (0.1M NaCl, pH 7.5, 0.5 M Tris-HCl, 0.001M EDTA). Precipitation and resuspension procedures were the same as described above for TNA extraction from herbaceous hosts.

Reverse transcription, synthesis of complementary DNA (cDNA) and polymerase chain reaction (RT-PCR): Reverse transcription was carried out with 1 µl of hexanucleotide random primers (0.5 µg/µl concentration), 5 µl of 1:10 diluted plant extract or TNAs and first strand buffer (Gibco BRL, Life Technologies) in total volume of 30 µl of solution at room temperature. Complementary DNA synthesis was performed essentially as described by Hadidi and Yang (1990). Reaction mixture was prepared in a volume of 20 µl, containing 4 µl 5x first strand buffer, 5 µl 0.3 M 2-mercaptoethanol, 2.5 µl 10 mM dNTP, 2.4 µl 0.1 M DTT and 0.8 µl cloned M-MLV RT (Gibco BRL, Life Technologies) and cDNA was synthesized at 37 °C for 1 h.

Forty five µl of PCR mixture containing C395 and H122 GVD-specific primers, *Taq* DNA polymerase enzyme and 1/10 aliquot of cDNA reaction were mixed and amplified in a DNA thermocycler (Perkin-Elmer Cetus). PCR amplification profile was performed at an annealing temperature of 48 °C.

Immunocapture (IC)-RT-PCR: IC-PCR was carried out according to Nolasco et al., (1993). Coating protocol was as described in Clark and Adams (1977). Polyproplyene microtubes (0.5 ml) were separately pre-coated with protein A at a concentration of 5 µg/µl or 2 µg/µl in coating buffer for 2 h at 37 °C. Fifty µl of crude sample extract prepared as described above were added to the tubes that had been previously coated with 200 µl of GVD polyclonal antiserum per tube (1:10 dilution in coating buffer) and incubated overnight at 4 °C. After through washing, 50 µl of cDNA reaction mix (Minafra and Gallitelli, 1995). cDNA was amplified with the same RT-PCR profile as mentioned in the previous section.

Polyacrylamide gel (PAGE) analysis and silver staining of PCR products: Aliquots of the PCR amplified cDNA fragments were analyzed by electrophoresis through 5% polyacrylamide vertical slab gel. Amplified DNA products were visualized with silver staining technique.

Electron microscopy: *In vitro*-grown shoot-tips from GVD positive grapevines were macerated in 0.01M potassium phosphate buffer, pH 7.0, 2.5% nicotine and mechanically inoculated onto *Nicotiana clevelandii* for electron microscopy observation. Leaf dips (Milne and Luisoni, 1977) from virus-infected *N. clevelandii* plants were negatively stained with 2% aqueous uranyl acetate and observed with a Philips 201C electron microscope.

Southern blot hybridization: The assay was carried out as described by Sambrook et al., (1989). A digoxygenin (dig)-DNA labeled probe from clone pGD8dt (Abou-Ghanem et al., 1997) containing 700 bp amplified sequence of GVD was used. Ten µl of amplified PCR products were electrophoresed in 1.2% agarose gel and stained in etidium bromide solution. Nucleic acids were tranferred onto Nylon Hybond N+ membrane (Amersham) through capillarity. Hybridization was performed for 16 h at 45 °C. Chemiluminescent detection was carried out to detect the dig-labeled probe according to manufacturer's handbook (Boehringer, Manheim).

RESULTS and DISCUSSION

GVD detection with RT-PCR in grapevines: GVD was first identified in in vitro-grown *V. vinifera* cv. Primus showing RW symptoms. Based on this findings, grapevine samples were randomly chosen from vineyard-grown vines with and without RW. All collected samples were tested by RT-PCR. Table 3 shows that 12 out of the totality sampled were positive for the virus in PCR. The size of the amplified GVD cDNA fragment was 700 bp as expected.

Table 3. Results of RT-PCR for the detection of GVD

Grapevine Samples	No.of tested Plants	No.of infected plants	%of incidence
Vines with RW	168	8	4.8
Vines without RW	75	0	0
cv. Primus with RW	50	4	8.0
Registered vines	14	0	0
Total	307	12	3.9

Eight of these positive plants were different grapevine varities (Table 4). The incidence of GVD in the grapevine with RW was 4.8% and 8.0% in cv. Primus. Neither registered nor RW-free vines proved to be infected by GVD. These results suggest that there is some correlation between GVD and RW disease.

Although GVD-infected grapevines were found, the virus was irregularly distributed in the vineyards. It was also observed that GVD had very low concentration in infected plants, as known from the light bands in polyacrylamide gel in Figure 1.

Table 4. Grapevine plants infected by GVD from the collection of the University of Bari

Sample	Variety	Origin
BA 41	Unknown	Unknown
MT 13	Italia	Italy
BU 6	Bolgar	Bulgaria
LE 12	Kosija Sisa-Bela	Italy
H 24	Gazin 1 (Aramon x Rupestris)	Hungary
H 27	Blue burgunder	Hungary
H 29	Regina dei vigneti	Hungary
H 32	Italian riesling	Hungary

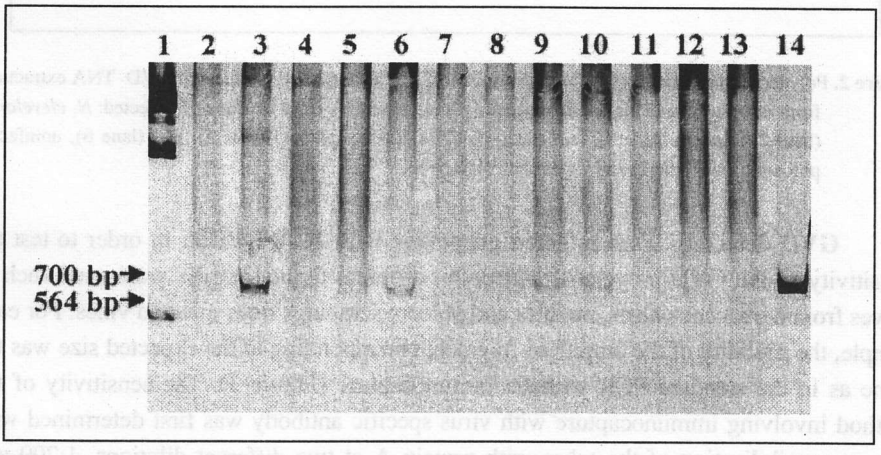

Figure 1. Polyacrylamic gel electrophoretic analysis of RT-PCR amplified products of GVD from infected grapevine phloem tissues. Marker λ *Hind* III (lane 1), different vines from cv. Primus at Castellanata (lane 2, 3, 4 and 5), vines with RW in the collection of the University of Bari (lane 6, 7, 8, 9, 10, 11, 12 and 13), positive control (lane 14).

Some GVD infected positive controls used for virus detection from herbaceous hosts and cv. Primus gave doubtful results by standard PCR. Therefore, it was necessary to check those samples with TNAs to be sure of the virus presence. According to banding patterns in polyacrylamide gels (Figure 2), the bands from herbaceous host (lane 2) were stronger than those from grapevine phloem extracts (lane 3 and 5). No amplification signals were obtained from leaf samples (lane 4) although further purification of TNAs was done with cellulose treatment (lane 6). These results were interpreted as an indication that GVD is irregularly distributed in infected vines.

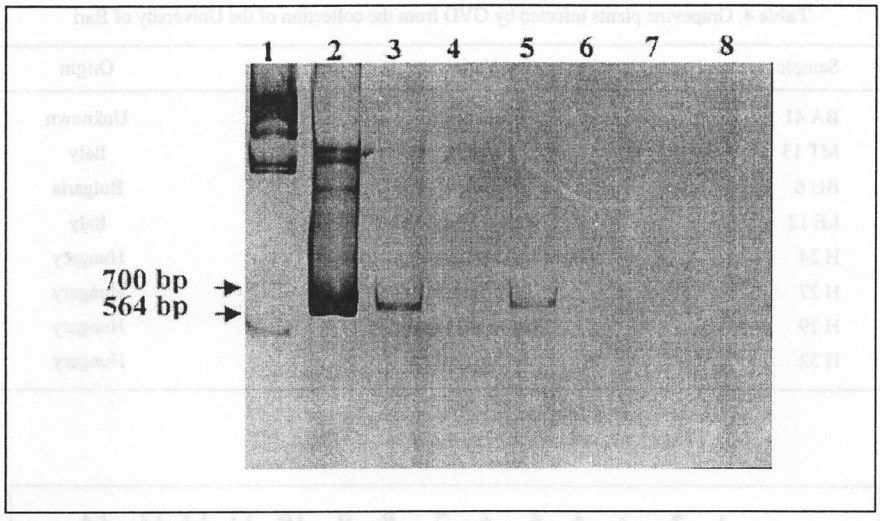

Figure 2. Polyacrylamic gel electrophoretic analysis of RT-PCR amplified products of GVD TNA extractions from infected grapevine and herbaceous plants. Marker λ *Hind* III (lane 1), infected: *N. clevelandii* (lane 2), phloem (lane 3), leaf (lane 4); CF11 treated: phloem (lane 5), leaf (lane 6), uninfected phloem tissue (lane 7) and water control (lane 8).

GVD detection from infected grapevine with IC-RT-PCR: In order to test the sensitivity of the GVD polyclonal antiserum, different tissue samples were used such as leaves from herbaceous hosts, petioles and phloem scrapings from infected vines. For each sample, the mobility of the amplified fragment corresponding to the expected size was the same as in the standard PCR without immunocapture (Figure 3). The sensitivity of the method involving immunocapture with virus specific antibody was first determined with the pre-sensibilization of the tubes with protein A at two different dilutions, 1:200 and 1:500 (lane 1 and 2), because the antiserum had a too low titer for direct serological diagnosis. Both protein A dilutions were successful for GVD detection in the petioles. Protein A at 1:500 dilutions was used in assays with herbaceous host extracts and grapevine phloem extracts undiluted or at dilutions of 1:5 and 1:10. The results showed that undiluted phloem extracts (lane 6) did not respond positively, contrary to the same samples dilution1:5 (lane 6) and 1:10 dilutions (lane 7). This was taken as an indication that undiluted extracts have an inhibitory effect on the assay. Infected herbaceous hosts were positive for GVD, but no amplification signals were obtained when IC-PCR was performed with healthy grapevine tissue (lane 8).

Electron microscope observation: Crude sap extract from *N. clevelandii* plants contained flexuous particles of identified GVD virions (Figure 4) with decoration tests. Virions showed were conspicuously cross-banding under electron microscope.

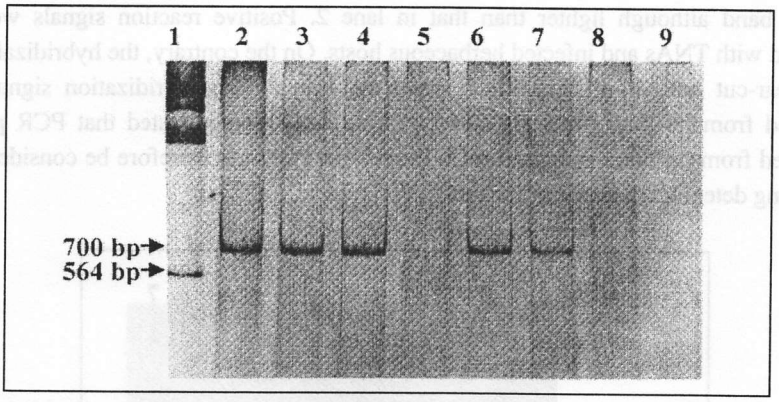

Figure 3. Polyacrylamic gel electrophoretic analysis of IC-RT-PCR amplified products of GVD from infected grapevine and herbaceous plants. Marker λ *Hind* III (lane 1), infected: petiole extracts with 1:200 and 1:500 dilutions of protein A (lane 2 and 3, respectively), herbaceous host (lane 4), undiluted phloem extract (lane 5), 1:5 and 1:10 diluted phloem extracts (lane 6 and 7, respectively), uninfected grapevine (lane 8) and water control (lane 9).

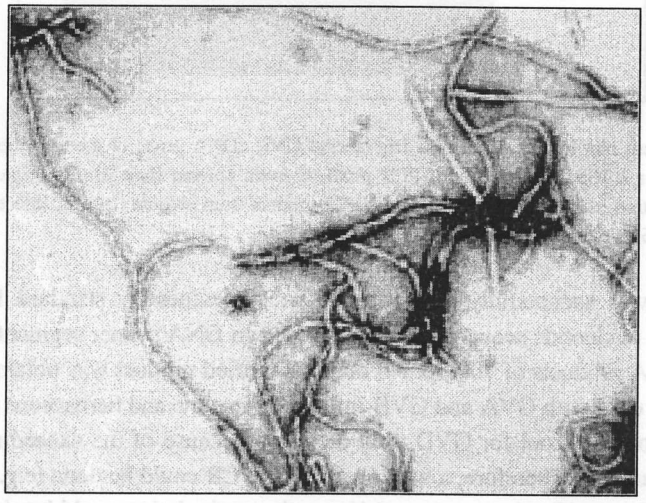

Figure 4. Leaf dip preparation from GVD-infected *N. clevelandii*. Bar = 100 nm.

Detection of GVD with molecular hybridization: The study of banding patterns of GVD was carried out using amplified DNA products from grapevine and herbaceous host, standard TNAs and IC-PCR. It was aimed at confirming the specificity of GVD bands after PCR analysis. The dig-DNA labeled probe recognized the respective amplified DNAs. Clear-cut hybridization signals were obtained with grapevine samples (Figure 5, lane 2) from the collection vineyard of the University of Bari. *V. vinifera* cv. Primus also

gave a band although lighter than that in lane 2. Positive reaction signals were also obtained with TNAs and infected herbaceous hosts. On the contrary, the hybridization was less clear-cut with IC-PCR products from cv. Primus. No-hybridization signals were observed from healthy grapevine controls. This assay demonstrated that PCR products amplified from different sources are GVD-specific. PCR can therefore be considered as a promising detection method for the virus.

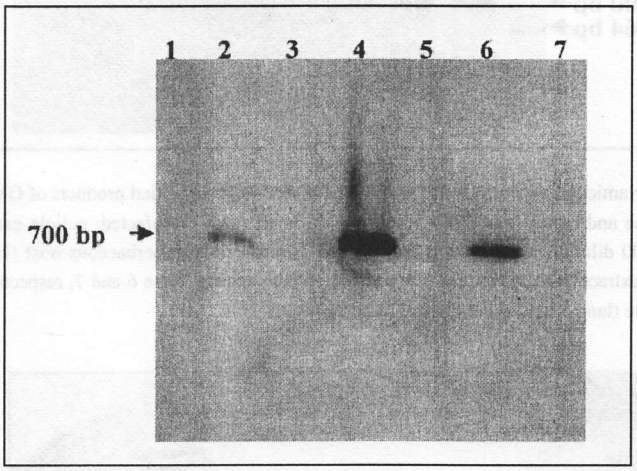

Figure 5. Southern blot hybridization with Dig-labeled GVD cDNA probe of the amplified PCR products. Marker λ HindIII (lane 1), RT-PCR products from: phloem (lane 2), cv. Primus (lane 3), TNA extraction from grapevine (lane 4), IC-PCR product from phloem (lane 5), infected *N. clevelandii* (lane 6) and uninfected grapevine (lane 7).

GVD was successfully was detected in grapevines by standard RT-PCR. The availability of nucleotide sequences enabled to design DNA primer segments that gave an amplified DNA products of 700 bp. No major amplified product was obtained with GVD primers when used with GVA and GVB-infected grapevine and herbaceous plant extracts. PCR was a powerful tool for GVD viral detection because of the exceedingly low viral titer in field samples. Therefore, a wider use of RT-PCR could be done (e.g. large scale of field surveys for certification programs) if sample manipulation could be simplified. PCR analysis of vines with and without RW symptoms, indicated that GVD occurs in Italy and other countries, and is associated with RW. However, with the present study no attempt was made to correlate GVD with any of RW disorders, as it is known for GVA and GVB which are already associated with KSG (Chevalier et al., 1993; Garau et al., 1994) and CB (Boscia et al., 1993; Garau et al., 1993), respectively.

The present investigation also demonstrated the successful application of RT-PCR for the detection of GVD RNA in total nucleic acid extracts from infected grapevine tissue

(phloem scrapings and petioles) and from herbaceous host tissue. Although further purification of TNAs was done from infected samples, no reliable and repeatable results were obtained from this type of source material.

IC-RT-PCR was applied with success for the detection of GVD in infected grapevine and herbaceous hosts. The protocol did not require any previous handling of plant tissue homogenates or thermal disruption of virions. It was possible to use higher quantity of crude sap extract than with standard PCR. Thus, it presented a further improvement of PCR technology.

Hybridization with non-radioactive-labeled probe could be an alternative for electrophoretic detection for PCR products. The use of dig-labeled probes is highly desirable because they are safe, do not have dangerous waste and can be stored for several months at -20 °C without appreciable loss of activity.

ÖZET

ASMA D VİRÜSÜ VE KIRIŞIK ODUN HASTALIĞI ARASINDAKİ OLASI İLİŞKİ

Kuzey İtalya'nın Apulia bölgesinde "mantarımsı kırışık odun" simptomu gösteren bir asmadan, bitki özsuyuyla taşınabilir yeni bir *Vitivirus*, asma D virüsü (ADV) izole edilmiştir. Virüse spesifik-DNA primerler, viral genomun 3' sonlu nükleotid dizininden hazırlanmıştır. Amplifiye olan DNA'nın büyüklüğü 700 baz çiftidir. Primerler, infekteli asma ve otsu konukçulardan elde edilen asma A ve B virüsü ekstraktları için çalışmamıştır. Asma D virüsünün varlığını araştırmak için farklı coğrafik bölgelerden ve farklı varyetelerden 307 örnek alınmış ve tersine transkripsiyon-polimeraz zincir reaksiyonlarıyla testlenmiştir. Ortalama virüs infeksiyonu kırışık odun simptomu gösteren asmalarda %4.8 ve cv. Primus'ta %8 bulunmuştur. Virüs infektesiz 89 asmada ADV bulunmamıştır. Viral partiküller (825 nm uzunluğunda, kıvrımlı iplik şeklinde), virüse spesifik antiserumla dekore edilmiştir. Antiserum asma A, B ve C virüsleriyle reaksiyon vermemiştir. ADV'nin DNA bantlarının spesifikliğini konfirme etmek için PCR ürünlernini, digoksijenle işaretlenmiş DNA probuyla Southern blot yönteminde hibridizasyonu yapılmıştır.

Anahtar Kelimeler: Asma, mantarımsı kırışık odun, Asma D virüsü

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J. Turk. Phytopath., Vol. 31, No. 3, 167-172, 2002 ISSN 0378 - 8024

Detection of Viroids in Grapevine Rootstocks by Sequential Polyacrylamide Gel Electrophoresis

Mona GAZEL Nüket ÖNELGE

University of Çukurova, Faculty of Agriculture, Plant Protection Department, 01330 Adana / TURKEY

ABSTRACT

Two possible sources responsible for the spread of grapevine viroids have been identified; 1) Mechanical transmission among vines which might be accomplished during routine cultural practices and 2) Systemic transmission between grafted rootstock and scion varieties containing grapevine viroids. Presence of grapevine viroids in rootstocks may function as reservoirs for viroid transmission. Grapevine viroids in rootstocks were detected by sequential polyacrylamide gel electrophoresis (sPAGE) in the laboratory. Grapevine yellow speckle viroid-1 (GYSVd-1), grapevine yellow speckle viroid-2 (GYSVd-2) and hop stunt viroid-grapevine (HSVd-g) were detected in analysed 140 Ru, 5 BB, 41 B, 110 R and 1103 P rootstocks.

Key words: Grapevine, Polyacrylamide, Electrophoresis, Viroid

INTRODUCTION

Grapevine is one of the most important crops in Turkey. Total production of grapevine in Turkey is 3.400.000 tons and it has 540.000 hectares growing area (Anonymous, 2000). Structural analysis of viroids isolated from grapevines has revealed that six distinct viroids are present in commercial varieties. Four major groups of grapevine viroids have been determined (Semancik and Szychowski, 1992). These are; i) Citrus exocortis viroid-grapevine (CEVd-g), a grapevine isolate of citrus exocortis viroid, ii) Grapevine viroid-cucumber (GVd-c), a grapevine viroid recovered from cucumber and Australian grapevine viroid (AGVd), iii) Grapevine yellow speckle viroid-1 (GYSVd-1) and Grapevine yellow speckle viroid-2 (GYSVd-2), two viroid inducing yellow speckle disease, iv) Hop stunt viroid-grapevine (HSVd-g), a grapevine isolate of hop stunt viroid.

Two grapevine viroids GYSVd-1 and GYSVd-2, have been implicated as the causal agents of yellow speckle (YS) disease. Since the other grapevine viroids don't cause any obvious symptoms, they don't evaluated as a disease agent up to now.

Symptoms of yellow speckle disease consistig of a few to a coalescence of chlorotic specks on leaves. These are mostly evident at the end of the summer (Stellmach and

Goheen, 1988) and this indicating that symptoms are strongly influenced by climatic conditions. Vein banding (VB) disease has been postulated to be either a late season expression associated with fanleaf degeneration caused by grapevine fanleaf virus (GFLV) (Martelli and Savino, 1988) or the response to coinfection by the YS disease agent and GFLV (Krake and Woodham, 1983). More recently, VB has been experimentally demonstrated to result from a synergistic reaction between grapevine viroids and GFLV (Szychowski et al., 1995).

In Turkey the studies on viroids of grapevine are limited. Önelge and Gazel, analysed 138 grapevine samples in the East Mediterranean region of Turkey. According to sPAGE and PCR analyses 43 of the 138 samples were found to be infected by one or more viroids. GYSVd-1, GYSVd-2, HSVd-g viroids and one viroid which has not been identified yet were determined. Stunting, yellow speckle and vein banding were observed as the most common symptoms of these infected grapevine samples (Önelge and Gazel, 2001).

The objective of this study was to determine the viroids of grapevine rootstocks (110 R, 1103 P, 5 BB, 140 Ru, 41 B) which are using prevailent in the Mediterranean Region.

MATERIALS and METHODS

Collection of grapevine rootstock tissues: Rootstocks which used in this study were collected from research and training farm of Çukurova University. Apex tissues of 140 Ru, 5 BB, 41 B, 110 R and 1103 P rootstocks were collected from actively growing plants in the summer of 2002. Visible symptoms were not shown on tested plants. From each rootstock 3 samples were taken for sPAGE analyses. Positive and negative control plants were taken from California University. Fresh tissues were used immediately or maintained at -20°C until extraction time.

Extraction of nucleic acids: Fresh or frozen tissue (5 g) was ground with a Virtis homogenizer in an extraction medium (5 ml) containing 0.5 M sodium sulfite, 1% sodium dodecyl sulphate and 4% 2-Mercaptoethanol. Water saturated phenol (18 ml) adjusted to pH 7.5 with 5M NaOH was added to the extraction medium.

The crude extract was centrifuged at 8.000 g for 20 minutes in 30 ml corex tubes. The aqueous phase was then made to 35% ethanol-STE (0.1 M NaCl, 0.05 M Tris-HCl and 1 mM EDTA, pH 7.2). To this solution, CF-11 cellulose (1 g) was added. The mixture was agitated by shaking or stirring overnight. Solution was loaded into the barrel of a 20 ml syringae and washed further with two or four void volumes of 30% ethanol-STE solution. The bound nucleic acids, including viroids and low molecular weight RNAs, were eluted with STE buffer and precipitated at -20°C for two to 18 hours with a minimum of 3 vol 95% ethanol after adjusting to 3 M sodium acetate (pH 5.5). The total

nucleic acid pellet was collected by centrifugation at 10.000 g for 20 minutes, dried in vacuo and resuspended in TKM buffer (0.01 M Tris-HCl, 0.01 M KCl and 0.1 M $MgCl_2$, pH 7.4). An equal volume of 4 M LiCl was added and the solution held at 4°C for four to 18 hours. After centrifugation at 10.000 g for 20 minutes, the 2M LiCl-precipitated nucleic acids were discarded while the LiCl-soluble nucleic acids were precipitated with a minimum of 3 vol 95% Ethanol and held at -20°C for two to 18 hours. The 2M LiCl-soluble nucleic acids were collected by centrifugation at 10.000 g for 20 minutes dried in vacuo and resuspend in TKM buffer (Szychowski et al., 1988).

Sequential polyacrylamide gel electrophoresis (sPAGE): Nucleic acids soluble in 2 M LiCl were analysed by sequential 5% PAGE under native and denaturing conditions containing 8 M urea. After electrophoresis in 5% polyacrylamide gel and staining with ethidium bromide, a section of the gel was excised and subjected to a second electrophoresis in 5% polyacrylamide containing 8 M urea. These conditions allow seperation of the characteristic circular and linear forms of the viroid molecular population (Rivera-Bustamante et al., 1986). Detection of the low concentrations of the viroid-like RNAs was made possible by silver staining (Igloi, 1983).

RESULTS and DISCUSSION

A total of 15 grapevine rootstock samples were analysed by sPAGE for the presence of grapevine viroids. As a result of sPAGE analyses, 10 rootstock of 5 varieties seen to be infected by one or more grapevine viroids (Table 1).

Table 1. Results of sPAGE analyses.

Rootstock varieties	Rootstock number		
	1	2	3
140 Ru	HSVd-g	HSVd-g	-
5 BB	GYSVd-1	-	HSVd-g
41 B	HSVd-g	GYSVd-1	GYSVd-1
110 R	-	HSVd-g	-
1103 P	-	GYSVd-1,GYSVd-2, HSVd-g	HSVd-g

There isn't any search about viroids in grapevine rootstocks in Turkey up to now. The result of this study showed that 6 rootstock plants were infected by HSVd-g, 3 plants infected with GYSVd-1. However, mix infection by 3 viroids was determined in 1103 P rootstock. No viroid bands were detected in other samples (Fig1).

Szychowski et al., analysed rootstock species to relate the occurrence and spread of the grapevine viroids in commercial rootstock sources and Vitis varieties. They analysed twelve rootstock and found four viroid pattern. These profiles were 1) GYSVd-1,

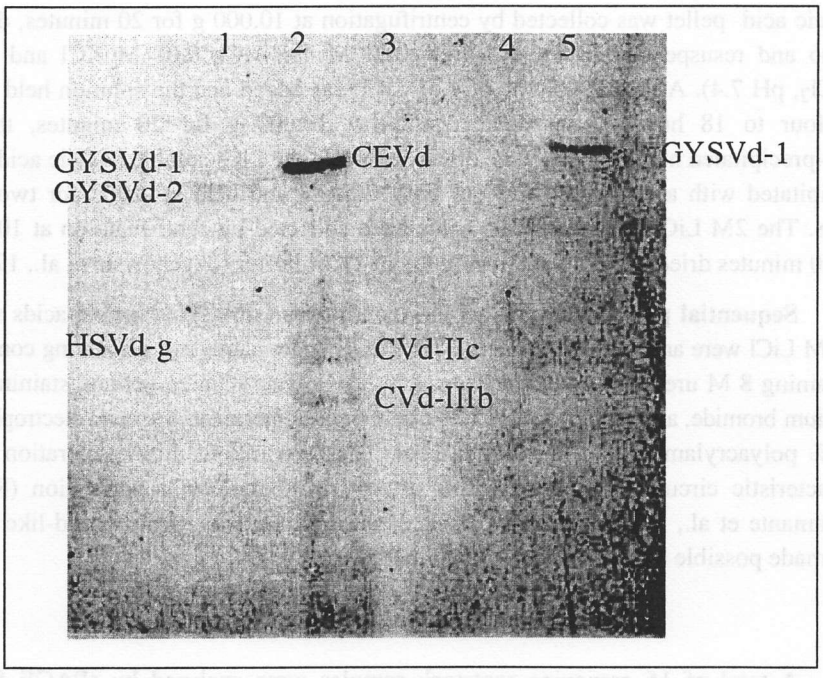

Figure 1. Field samples of grapevines determined by polyacrylamide (5%) gel containing 8M urea and stained silver after processing by sequential PAGE. Lane 1 Grapevine yellow speckle viroid-1 (GYSVd-1), Grapevine yellow speckle viroid-2 (GYSVd-2) and Hop stunt viroid-grapevine (HSVd-g) from field sample, Lane 2 Citrus exocortis viroid (CEVd), Citrus viroid IIc (CVd-IIc), Citrus viroid IIIb (CVd-IIIb) from citrus as a positive control, Lane 3 the sample of uninfected grapevine, Lane 4 and 5 grapevine samples infected with GYSVd-1.

GYSVd-2 and HSVd-g, 2) GYSVd-1 plus HSVd-g, 3) HSVd-g, 4) Viroid free. They found that wine and rootstock varieties contained a greater proportion of the more common GYSVd-1 plus HSVd-g viroid profile (Szychowski et al., 1991).

Duran-Vila et al. (1990), made a survey to evaluate the occurrence of grapevine viroids in 112 sources including the most representative wine and table grape varieties and rootstocks grown in Spain. They found 1 of the 10 rootstock sample was found to be viroid free. The other 9 rootstock samples were infected with GYSVd-1 and HSVd-g.

A possible factor contributing to the widespread occurrence of grapevine viroids in varieties is the presence of grapevine viroids in rootstocks, which may function as reservoirs for viroid transmission The presence of viroids in grapevine rootstocks increases the risk of spreading the viroids to vineyards (Szychowski et al., 1988).

Analyses of samples obtained from European sources indicated a similar pattern of viroids to that which was found in California sources (Semancik et al., 1987;

Szychowski et al., 1988). All samples received from France, Italy and Spain were found to contain at least one viroid. Also it is demonstrated that GYSVd-1 plus HSVd-g, the most common viroid profile found in California sources, was also predominant in European results (Szychowski et al., 1991). Analyses of grapevine scion and rootstock varieteis obtained from East Mediterranean region of Turkey indicated a similar pattern of viroids found in California and Europe. The results of sPAGE showed that GYSVd-1 and HSVd-g were common viroids in grapevine rootstock varieties in East Mediterranean region.

This is the first report of GYSVd-1, GYSVd-2 and HSVd-g in analysed 140 Ru, 5 BB, 41 B, 110 R and 1103 P grapevine rootstocks in Turkey.

ÖZET

ASMA ANAÇLARINDA VİROİDLERİN POLİAKRİLAMİD JEL ELEKTROFOREZ YÖNTEMİ İLE TANILANMASI

Asma viroidlerinin yaygın olmasında iki olası kaynak söz konusudur. Bunlardan birincisi rutin kültürel uygulamalar esnasında bağlar arasındaki mekanik taşınma, ikincisi ise asma viroidlerini içeren anaç ve kalem arasındaki sistemik taşınmadır. Anaçlarda asma viroidlerinin varlığı viroid taşınması için kaynak olarak rol oynamaktadır. Asma anaçlarındaki viroidler laboratuvarda sequential poliakriakrilamid jel elektroforez (sPAGE) yöntemiyle tanılanmıştır. Asma sarı benek viroidi-1 (GYSVd-1), asma sarı benek viroidi-2 (GYSVd-2) ve şerbetçi otu cüceleşme viroidi-asma (HSVd-g) analiz edilen 140 Ru, 5 BB, 41 B, 110 R and 1103 P anaçlarında ortaya koyulmuştur.

Anahtar Kelimeler: Asma, Poliakrilamid, Elektroforez, Viroid

ACKNOWLEDGEMENTS

We are grateful to Dr. Semih TANGOLAR for kindly supplying the rootstocks.

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J. Turk. Phytopath., Vol. 31, No. 3, 173-183, 2002 ISSN 0378 - 8024

dsRNA Analysis of Turkish Beet Necrotic Yellow Vein Virus (BNYVV) Isolates[#]

Filiz ERTUNÇ* **Diğdem İLHAN****

* Ankara University Faculty of Agriculture Department of Plant Protection 06110 Dışkapı / Ankara / TURKEY

** Plant Protection Central Research Institute Bağdat Caddesi No: 250 P.O. Box: 49 06172 Yenimahalle / Ankara / TURKEY

ABSTRACT

In this research, 73 rhizomania infected soil samples were collected from Kastamonu and Turhal sugar refinery areas and sugar beet cv. Fiona has been sown as a trap plant for the propagation of BNYVV (Beet Necrotic Yellow Vein Virus). dsRNA analysis has been done to all of the samples collected and best results has been obtained by using Sabanadzovic and diTerlizzi method.

Only in 34 of the isolates dsRNA profiles were determined. But no result was obtained from the remaining 41 isolates. RNA1, the largest RNA of the virus, was determined in 13 isolates, RNA3 in 1 isolate, and RNA4, the smallest part of the genome, in 4 isolates. RNA1+2 were together in 6 isolates and RNA 1+3 in 4 isolates. RNA 1+4 were detected in 6 isolates and in 1 isolate RNA 1+2+3 was present. No result was obtained about the presence of RNA5 in our research.

Key words: Beet Necrotic Yellow Vein Virus, BNYVV, dsRNA analysis

INTRODUCTION

Sugar beet (*Beta vulgaris* L. var. *saccharifera*) is an important industrial plant belonging to Chenopodiaceae family. It is especially cultivated in Thrace, Western and Central Black Sea, Middle Anatolia, and Eastern Anatolia provinces in Türkiye (Erdiller and Özgör, 1994; Ertunç et al., 1998).

The most important viral disease in our country that limits sugar beet production is Beet necrotic yellow vein virus (BNYVV), known as rhizomania. The disease is called as "root madness" in literature. The disease is a virus-fungus complex; the virus is Beet necrotic yellow vein virus (BNYVV); and fungus is *Polymyxa betae* Keskin (Plasmodiophoramycetes-Protozoa), which is the vector of the virus at the same time (Keskin, 1964).

[#] This manuscript is a part of TUBITAK-TOGTAG project no. 1709.

dsRNA ANALYSIS OF TURKISH BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) ISOLATES

BNYVV is a member of furovirus genus which is rod shaped, + stranded, and has a multipartitate genome organization. It was first determined in Italy by Canova in 1959 and Tamada named it as Beet necrotic yellow vein virus (BNYVV) in 1975 in Japan (Tamada and Baba, 1973; Tamada, 1975). Vector fungus was identified by Keskin (1964) and infection of root and structures formed by the fungus were observed by him. Fungus forms plasmodium following the root infection and these plasmodial structures turn into zoosporangia, which contains dormant zoospores, or cystosori, including resting spores inside. BNYVV causes severe yellowing of leaves and/or chlorosis in veins (Whitney and Duffus, 1991). Later, veins become typically necrotic, which gives the virus its name. The main damage of the causal agent occur on the under ground organs of the plants. After the infection, proliferation of the roots was observed. Infected roots cannot grow up thug loose their marketing value. The disease also causes necrosis in xylem. It is especially widespread under high moisture conditions.

Rhizomania was first determined in Türkiye in Alpullu Sugar Refinery area but the first data about the disease was published by Vardar & Erkan (1992). It is also one of the most important problems of sugar beet production in European countries, China, Japan and USA (Tamada, 1975; Putz et al., 1990; Rush and Heidel, 1995).

BNYVV can serologically be determined and also studies are performed on its RNA profiles and sequence analysis. It has been found out that the isolates, which have different RNA components, also differ in pathogenesis (Bouzoubaa et al., 1986; Koenig et al., 1984). The PCR products are also determined, and it is published that two different strains (A and B) exist and the Turkish isolate sent was included in A strain (Kruse et al., 1994). There is no difference in nucleotide sequence of these two strains, so they serologically do not differ.

BNYVV has four different RNA components named as RNA 1, 2, 3, and 4. But in the latest researches in some Japanese isolates the 5th RNA, which is 1349 nucleotide (nt) long, is declared (Saito et al., 1996; Koenig et al., 1997). RNA1 is the biggest component of viral genome and is 6746 (nt) long. It encodes a few large polypeptides whose functions are unknown. It is also responsible for virus replication. RNA2 is 4612nt long and contains the coat protein and code of 85kd large polypeptide. This polypeptide acquires the immunologic reaction of antiserum and the virus. RNA1 and 2 together are responsible for viral assembly and cell-to-cell movement. RNA3 is 1774nt long and is responsible for symptom formation in both sugar beet and indicators (Kuszala et.al., 1986). Absence or deletion of RNA3 causes mild symptoms on plants. RNA 4, which is 1465-1467nt long, plays an active role in transmission of BNYVV by *Polymyxa betae* with RNA3.

During the replication in plant cells, dsRNAs occur as intermediate product in single stranded virus infection, but in healthy plants dsRNA formation does not occur. The number and largeness of dsRNAs formed are used as criteria in determination of

pathogen virus. dsRNAs in infected plants are signs of an ssRNA virus in the plant and replicative form of it or it is an evidence for dsRNA infection in the plant (Dodds and Barr- Joseph, 1983; Dodds et al., 1984).

Morris and Dodds have performed the first studies about dsRNA in 1979 (Morris and Dodds, 1979). They have developed a dsRNA analysis method for *Ustilago maydis* and *Endothia parasitica* fungal tissues. This method was later on improved by Valverde et al. (Valverde et al., 1990).

In this research, our aim was to determine the RNA profile of the virus and getting information about molecular configuration of Turkish BNYVV isolates. It is a lack in the world literature and it is also targeted to eliminate this deficiency.

MATERIALS and METHODS

Virus Culture

Soil samples infected with BNYVV were collected from Kastamonu and Turhal sugar refinery areas in 1994. Each sample was taken from 10 different fields and mixed and they were placed into polyethylene bags which contained double holes of 0.5 cm in diameter at the bottom. Later on they were placed onto pots and 10 seed of sugar beet cv. Fiona were sown into them. The plants were maintained in a greenhouse for 3 months with a temperature range of 25±2°C. Then they were harvested and washed with tap water to clean up from soil particles. Later, they were placed into polyethylene bags with labels and were stored in -25°C until dsRNA analysis was performed. The tests were performed in the year of 2000.

Biologic Index

The trap plants were inoculated to *Chenopodium quinoa* to perform biological indexing. 1 g leaf sample was homogenized with a mortar and pestle in 0.1 M sodium phosphate buffer (pH 7.0, containing %2-mercapto ethanol). The sap was inoculated to *C. quinoa* leaves and 10 days after the inoculation symptoms were observed and recorded.

dsRNA Analysis

Three different methods were experimented and compared for dsRNA analysis which were Morris & Dodds (1979), Valverde et al. (1990) and Sabanadzovic and diTerlizzi (1994).

Morris & Dodds (1979) Method

10 g of frozen plant tissue was homogenized with 10 ml GPS (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, pH 9.5) buffer, 10 ml 10 % SDS, 0.1 ml mercapto ethanol, 10 ml water-saturated phenol (including 0.1% 8-hydroxyquinoline) and 10 ml chloroform, isoamyl alcohol (24:1) The mixture is stirred on ice for 30 minutes and centrifuged for

20 minutes at 8000g. Absolute ethanol was added to the supernatant until 15% final concentration. Whatman CF-11 was added on it (0.25g/20ml). It is stirred on ice for 10 minutes and centrifuged 10 minutes at 6000 g. The pellet was collected and resuspended with 5 ml STE (1 M NaCl, 0.5 m Tris, 0.01 M EDTA) /15% ethanol. Cellulose was placed into a column and ssRNAs were removed by washing the column with 60 ml STE/ethanol. After adding 5 ml STE to the column, dsRNAs were collected in a tube. 0.003 M $MgCl_2$ and 10 µl/ml DNAse were added and incubated at 30°C. Then the filter was precipitated with equal volume of ethanol and centrifuged 15 minutes at 10.000g. The precipitate of dsRNA was stored at - 20°C.

Valverde et al. (1990) Method

3.5 g of plant tissue was homogenized with 6 ml 1XSTE buffer and taken into a centrifuge tube of 50 ml. 1 ml 10% SDS, 0.5 ml of 2% bentonite solution and 9 ml STE-saturated phenol was added. The mixture was stirred for 30 minutes and centrifuged for 15 minutes at 8000 g. 10 ml of the supernatant was taken to another centrifuge tube. 2.1 ml of 95% ethanol was added to each tube, mixed well and incubated at 4°C overnight. For each sample 1 g of cellulose was placed in 50 ml centrifuge tubes and 1XSTE/ 16% ethanol was added. 2 columns were prepared with filters and fibreglass and STE was added to the drained column. The sample was added to the first column and the liquid was thrown out. Column was washed with 40 ml 1XSTE/%16 ethanol and was drained with 2.5 ml 1XSTE. Then 10 ml of 1XSTE was added, the liquid was taken to a centrifuge tube of 50 ml and 2 ml 95% ethanol was added. The sample was added to the 2nd column and the liquid was thrown out. Column was drained with 2.5 ml 1XSTE. Later, 6 ml 1XSTE was added and the liquid was taken to a centrifuge tube. 0.5 ml sodium acetate and 20 ml 95% ethanol was added and stored at -20°C for at least 2 hours to precipitate dsRNAs. Then it was centrifuged 25 minutes at 8000g, the supernatant threw out and the tube was turned down and dried for 15 minutes. The pellet was resuspended in 200 µl EG buffer and used in electrophoresis.

Sabanadzovic and diTerlizzi Method

20 g of plant tissue was homogenized in E buffer. 25 ml water-saturated phenol and 25 ml chloroform: isoamyl alcohol (24:1) was added and mixed at room temperature for 45 minutes. The mixture was centrifuged at 10.000 g for 10 minutes and the supernatant was collected. Absolute ethanol (17% final concentration) and Whatman CF-11 cellulose (1g/10 g plant tissue) was added. The mix was stirred at least for 1 hour at room temperature and centrifuged at 10.000 g for 10 minutes. Cellulose was resuspended with 1XSTE/17% ethanol and removed by repeated centrifuges at 10.000 g for 5 minutes. Cellulose was placed onto a column with filter paper and fibreglass and was washed with 12 volumes of STE/17% ethanol. dsRNAs were washed with 5 vol of 1XSTE. dsRNAs were precipitated with 5 vol of cold absolute ethanol and 0.1 vol of 3M sodium acetate pH 5.5 and incubated at -20°C overnight. It was centrifuged at 10.000 g for 25

minutes and the pellet was collected. It was resuspended with 1 ml TE buffer pH 5.5. 2.5 vol of cold absolute ethanol and 0.1 vol of 3M sodium acetate pH 5.5 was added and incubated at -20°C overnight. Then it was centrifuged at 15.000g for 20 minutes and the pellet was collected. The pellet was washed with 70% ethanol and centrifuged at 15.000 g for 5 minutes. Pellet was resuspended in sterile bidestilated water and used in electrophoresis.

Electrophoresis

Electrophoresis was performed in 1% agarose gel for 1 hour. Later on the gel was stained with ethidium bromide and was observed in a UV transilluminator and the photos were taken by a polaroid camera.

RESULTS and DISCUSSION

Sugar beet cv. Fiona plants in the greenhouse showed typical symptoms of rhizomania, which are proliferation of rootlets, beard in roots, chlorosis of leaves and necrosis of veins (Fig. 1).

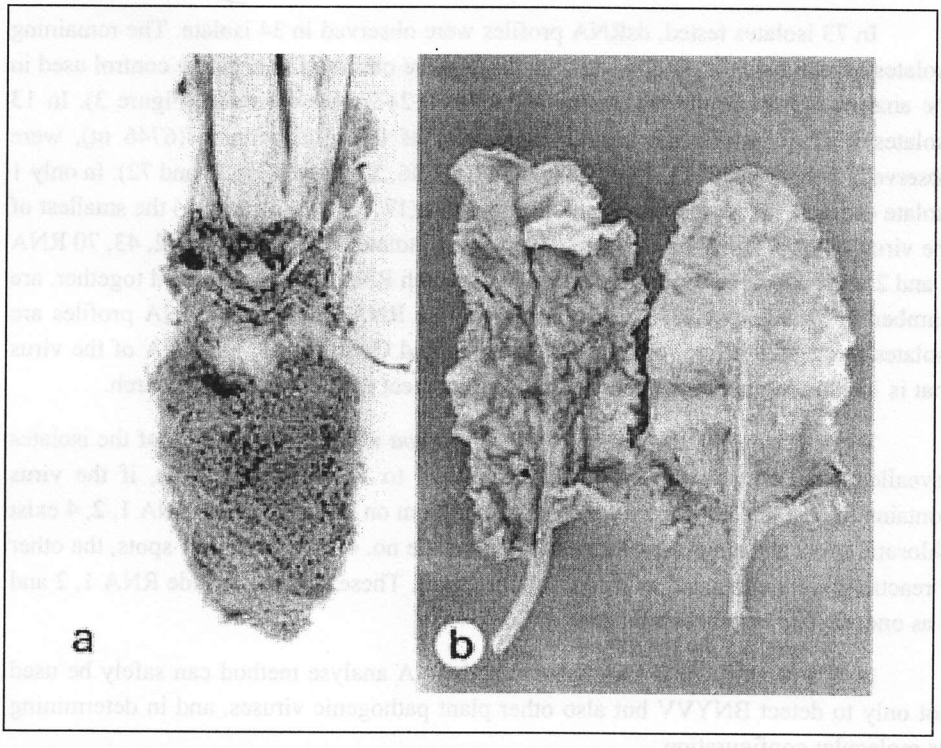

Figure 1. Typical symptoms of rhizomania. a. intensive bearding of roots and b. yellowing and necrosis on leaves.

All the isolates used in the research were mechanically inoculated to *C. quinoa*, but only 8 of them showed symptoms on the host (isolates no. 4, 15, 17, 21,26, 50, 57, 58), no results were observed on the other isolates. The first symptoms on *C. quinoa* were observed 10 days after the inoculation and were generally appeared as chlorotic local spots. Only isolate no. 4 has showed the yellow spot symptom. The main reason is thought to be the deletion of RNAs during the passages to *C. quinoa* (Brunt et al., 1996; Putz et al., 1990; Lemaire et al., 1988).

In 8 isolates that have given negative results in I-ELISA tests, which was performed in a previous project, dsRNA profiles were determined (isolates no. 3, 5, 25, 30, 31, 39, 46, 49). We think this may be because of low virus concentration in the plants. According to this data, we can say that dsRNA analysis is more sensitive when compared to I-ELISA test.

According to the results of three different dsRNA analysis methods, best results were obtained by Sabanadzovic and diTerlizzi method (1994) in the preliminary tests. Therefore this method was chosen for this research.

In 73 isolates tested, dsRNA profiles were observed in 34 isolate. The remaining isolates had no dsRNA. And no dsRNA bands were observed in negative control used in the analysis. Only in one isolate (no. 4) RNA 1+2+3 were observed (Figure 3). In 13 isolates RNA 1, which the largest component of the viral genome (6746 nt), were observed (isolates no. 3, 15, 18, 20, 34, 44, 48, 49, 56, 58, 60, 69 (Fig 2) and 72). In only 1 isolate (no. 67) RNA3 (1774 nt), and in 3 isolates (17, 31 (fig. 2), 50 no.) the smallest of the virus, RNA 4 (1465-67 nt) were observed. In isolates no. 19, 21, 39, 42, 43, 70 RNA 1 and 2 were observed together. The isolates, which RNA 1 and 3 observed together, are number 5, 11, 25, and 27. The isolates that have RNA 1+4 in their RNA profiles are isolates no. 2, 27, 30, 46, 52, and 71. In Japan and Germany the 5th RNA of the virus that is 1348 nt log is determined. But we did not meet this RNA in our research.

When compared, the symptoms on *C. quinoa* and dsRNA profiles of the isolates revealled parallelity to the literature. According to Brunt et al. (1996), if the virus contains RNA 1+2+3, it shows yellow spot symptom on the host, and if RNA 1, 2, 4 exist chlorotic spots and rings are observed. Only isolate no. 4 showed yellow spots, the other 7 reactions were evaluated as chlorotic local spots. These isolates include RNA 1, 2 and 4 as one, double or trial combinations.

In this research, it is concluded that dsRNA analyse method can safely be used not only to detect BNYVV but also other plant pathogenic viruses, and in determining its molecular configuration.

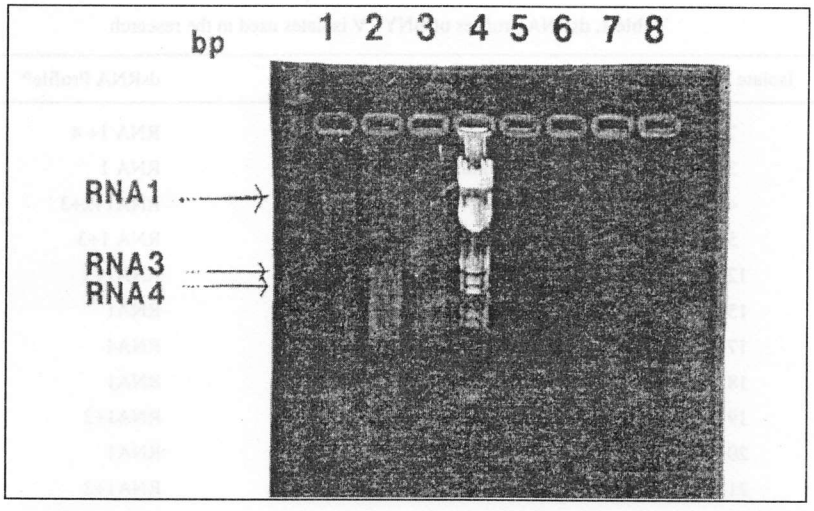

Figure 2. dsRNA profiles of some isolates used in the research

1) Isolate no. 69 (Turhal), 2) Isolate no 68 (Turhal), 3) Isolate no 67 (Turhal), 4) (λ DNA/Eco RI+Hind III), 5) Negative Control, 6) Isolate no 2 (Kastamonu/Taşköprü), 7) Isolate no 55 (Turhal/ Niksar), 8) Isolate no 31 (Kastamonu).

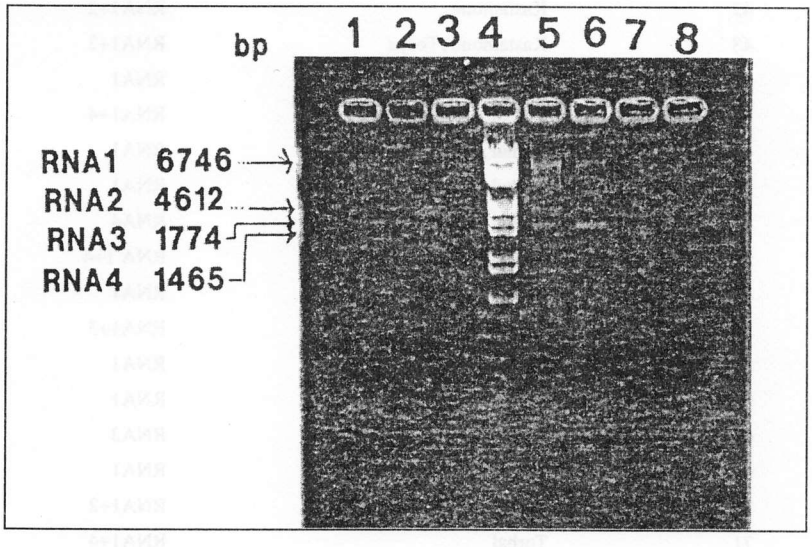

Figure 3. dsRNA profiles of some isolates used in the research

1) Isolate no. 52 no (Turhal/Artova), 2) Isolate no 22 (Turhal), 3) Isolate no 8 (Turhal/Pazar), 4) (λ DNA/ EcoRI+Hind III), 5) Isolate no 4 (Kastamonu/Taşköprü), 6) Isolate no 30 (Kastamonu), 7) Isolate no 32 (Kastamonu), 8) Negative Control.

Table 1. dsRNA profiles of BNYVV isolates used in the research

Isolate Number	Province	dsRNA Profile*
2	Kastamonu /Taşköprü	RNA 1+ 4
3	Kastamonu /Taşköprü	RNA 1
4	Kastamonu /Taşköprü	RNA1+2+3
5	Kastamonu /Tosya	RNA 1+3
12	Turhal/Pazar	RNA1+3
15	Turhal/Pazar	RNA1
17	Turhal/Pazar	RNA4
18	Turhal/Pazar	RNA1
19	Turhal /Zile	RNA1+2
20	Turhal /Merkez	RNA1
21	Turhal/ Zile	RNA1+2
25	Turhal/Zile	RNA1+3
27	Kastamonu	RNA 1+4
30	Kastamonu	RNA 1+4
31	Kastamonu	RNA 4
34	Kastamonu	RNA1
39	Kastamonu /Boyabat	RNA1+2
42	Kastamonu	RNA1+2
43	Kastamonu /Tosya	RNA1+2
44	Turhal /Artova	RNA1
46	Turhal/Çamlıbel	RNA1+4
48	Turhal /Artova	RNA1
49	Turhal /Artova	RNA1
50	Turhal /Artova	RNA4
52	Turhal /Artova	RNA 1+4
56	Turhal /Merkez	RNA1
57	Turhal/ Zile	RNA1+3
58	Turhal	RNA1
60	Turhal /Zile	RNA1
68	Turhal	RNA3
69	Turhal	RNA1
70	Turhal	RNA1+2
71	Turhal	RNA1+4
72	Turhal	RNA1
73	Negative Control	-

(-), shows no dsRNA profiles.

ÖZET

TÜRK ŞEKER PANCARI NEKROTİK SARI DAMAR VİRÜSÜ İZOLATLARININ dsRNA YÖNTEMİ İLE ANALİZİ

Bu çalışmada, Kastamonu ve Turhal Şeker Fabrikaları ekim alanlarından rhizomania ile bulaşık 73 toprak örneği alınmıştır. Bu topraklar üzerine tuzak bitki olarak Fiona çeşidi şeker pancarı tohumları ekilmiştir. Toplanan tüm örnekler dsRNA analizine tabi tutulmuş ve en iyi sonuçlar Sabanadzovic and diTerlizzi metodundan elde edilmiştir.

İzolatların yalnızca 34'ünde dsRNA profili tespit edilmiştir. Kalan 41 izolatta ise herhangi bir dsRNA varlığı gözlenmemiştir. Virüsün en büyük komponenti olan RNA 1, 13 izolattta saptanırken, RNA 3, 1 izolatta, en küçük komponent olan RNA 4 ise 4 izolatta tespit edilmiştir. RNA1+2, 6 izolatta birlikte bulunurken, RNA 1+3 4 izolatta tespit edilmiştir. RNA 1+4 6 izolatta ve RNA 1+2+3 1 izolatta gözlenmiştir. Çalışmada RNA5'in varlığına rastlanmamıştır.

Anahtar Kelimeler: Şeker Pancarı Nekrotik Sarı Damar Virüsü, BNYVV, dsRNA analizi

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