ISSN 0378 - 8024

THE JOURNAL OF TURKISH PHYTOPATHOLOGY

PUBLISHED BY THE TURKISH PHYTOPATHOLOGICAL SOCIETY

Volume : 29 Number : 2-3 May-September : 2000

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. 29 May - September NO. 2-3

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Prof. Dr. Gürsel ERDILLER
(1942 - 2000)

Prof. Dr. Gürsel Erdiller was born in 1942. She has graduated from Ankara University Faculty of Agriculture in 1965 and has started as assistant of Plant Protection Division at the same year. She was awarded with PhD in 1970 and promoted to Associate Professor degree in 1975. She has been as post graduate in Germany (Braunschweig) for 2 years between 1975-1977 by Göttingen linkage project of Ankara University. She has promoted to full Professor degree in 1982 Phytopathology Section of the same department. She has been the head of the Plant Protection Department for three years between 1994-1997. She has supervised plenty of MSc and PhD students and has prepared totally 94 scientific publications, proceedings and books. She was married and has 2 daughters. She has died in December 7th 2000.

J. Turk. Phytopath., Vol. 29, No. 2-3, 53-60, 2000 ISSN 0378 - 8024

The Incidence and Distribution of *Colletotrichum coccodes* (Wallr.) S.J. Hughes at Stored Potato Tubers and Reactions of Various Potato Varieties Against the Pathogen at Central Anatolia Region

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ABSTRACT

Studies have been carried out in 1990-1997. Surveys were done at farmers storages in Bolu, Nevşehir and Niğde provinces and the tubers for propagating or consumption or the tubers imported by the private sector for production purposes were investigated for the disease. The average incidence and distribution rate of the fungus for the region were found to be 2.61 and 51.47% respectively. In variety reaction studies, all varieties were infected by the fungus and wound diameter on tuber was 0.38-1.32 cm depending on the varieties. Surface inoculations were failed to develop the mycelium growths.

Key words: Potato, *Colletotrichum coccodes*, Varietial resistance

INTRODUCTION

Bolu, Nevşehir and Niğde provinces share the 31.64% of potato cultivation area and 46.33% tuber production of the whole country (Anonymous, 1999). *Colletotrichum coccodes* is one of the important disease that affects the tubers at storages as well as in fields.

The pathogen is soil borne and infectious on potato roots, stolons and tubers, also causing symptoms on foliage (Dickson 1926, Pavlista and Kerr 1992, Johnson and Miliczky 1993, Johnson et al., 1997). Tubers have a great importance at the distribution of the agent. As symptoms, the agent causes paleing and wilting beginning from the top of the plants continuing toward downwards, also inducing dry rotting at root crown. Although this development of the disease matches those caused by *Fusarium* and *Verticillium*, in this case it is more rapid and on the contrary of *Verticillium*, the plants die at the advanced stages of the disease. Sclerotia are produced on the stem. Underground stem turns to color of purple. The pathogen is transmitted to the storage with the stolons on the tubers or mainly on the tuber surface. Tuber infection appears as brownish to gray discoloration over a large portion of the tuber (Zitter et al., 1989).

There are several studies on the disease agent, *C. coccodes*, carried out in Turkey (Karaca 1964, Döken 1977, Turkensteen 1985, Demirci ve Döken 1989, Turak ve Hantaş 1992, Boyraz ve Yıldız 1998). Turkensteen (1985) had been detected the pathogen on 2 samples 20 from Bolu, and on 1 sample 30 from Nevşehir. In this research, information is cited on the incidence and distribution of the disease in stored planting material tubers for propagating or consumption at farmers storages, or the tubers imported by the private sector for production purpose in Bolu, Nevşehir and Niğde provinces and also on reaction of potato varieties.

MATERIALS and METHODS

Survey Studies

Studies had been carried out at farmers storages in Bolu, Nevşehir and Niğde provinces in 1990-1994. Certificated tubers for planting or the others for consumption, or the tubers imported by the private sector as propagating material were investigated. Sampling sites and potato varieties have been listed in Table 1 and 2. Regarding the storage capacities, 200 tubers were taken for each 10 tons in each storage selected at random (Anonymous, 1976). Tubers with symptoms of disease or suspected for the pathogen were taken in Potato Dextrose Agar for the fungal growth. Incidence rate is calculated according to weighed mean, and the distribution area is formed as accepting the storages infected in case of the determination of the disease in samples (Bora ve Karaca, 1970).

Variety Reaction Studies

The method cited by Boyd (1972) was used. *C. coccodes* developed in PDA medium and sclerotia comprising parts of the fungus was cut out in 6-mm diameter disks with agar drillers. Tubers were treated with 95% ethyl alcohol, disked out with an another driller, the previous disc consists of fungal part placed in the tuber and the holes were closed with disk drilled out from the tubers. In the inoculations made without disk removing from tuber, the inoculums were put on the surfaces of the tubers. For each variety, two groups were formed with 10 tubers and control (each tuber being one replicate) and left for fungal development at 22 and 4°C for 30 days. In evaluation, the dimensions of wounds caused by the pathogen on tubers were measured. Data were analyzed using analysis of variance. The difference was evaluated by Duncan's multiple rang test.

Varieties used in the trials were supplied from the Seed Registration and Certification Institute.

RESULTS AND DISCUSSION

Survey Studies

An amount of 561.550, 304.800 and 43 ton tuber were investigated in the storages of Bolu, Niğde and Nevşehir provinces, respectively. The incidence and the distribution of the disease were found to be 0.51 and 54.71% in Bolu, 0.99 and 21.94% in Niğde, 6.33 and 77.77% in Nevşehir respectively (Table 1).

An amount of propagating material tuber of 6015.5 ton imported by private sector was also investigated. An amount of 5594.5 t tuber belonging to the varieties Planta, Shepody, R.Burbank, Ausonia, Scala, Semena were found to be free of the disease. However, an amount of 421 t tubers belonging to the varieties Granola, Panda, Herta, Hermes, Ernst Tolz, Agria, Apollo, Resy, Marfona and Planta were found to be infected in a rate differing between 1-30% (Table 2).

The disease is widespread nearly at all countries (Dickson 1926, Stevenson et al 1976) and nearly not exists in the quarantine and certification lists. Besides, Turkey has not a well defined propagating tuber production system and the import material have been usually contaminated with this pathogen. By the year 1984, huge amounts of propagating material tubers were imported in Turkey and the incidence and the distribution of the disease have been expected to be increased rapidly. Although it is not so far Bolu, it is in an increasing tendency for Nevşehir. Hence, although Turkensteen (1985) found 1 sample infected in 30, the rate was found to be 6.33% in this study. This increase may be caused from the effect of the climate and soil factors on the development of the disease. For example, Stevenson et al. (1976) reports that the disease developes more rapidly at the crops highly stressed with long period rains at the beginning, low temperatures followed by dry period. Otazu et al. (1978) informs that the pathogen likely to attack to the plants in stress because of the wilting agents, water stress, rough fields, low or high nitrogen levels, high temperatures, bad drainages.

Variety Reaction Studies

The results of the studies done by drilling the tubers for inoculation and left for infection at 22°C are given at Table 3. No fungal development was determined at the surface inoculation group, 4°C and control.

Although there are variations in reaction status regarding the isolates used, all varieties was found to be affected by the fungus in varying degrees. At the first group stands Agria with 1.32 cm fungal growth. Marfona, Fienna and Marabel formed the second group where Cosmos, Resy and Agata were in third. Lola, Donella in fourth and Granola formed the last group with the least fungal development. In general, *C. coccodes* doesn't

Table 1. The incidence and the distribution of *Colletotrichum coccodes* in Bolu, Niğde and Nevşehir provinces in 1990-1994.

Province	Village	Disease rate %							Incidence	Distribution
District		Resy	Marfona	Ausonia	Granola	Cosima	İlona	Planta	Rate	Rate
BOLU	Doğancık	1,1,1,1,1*	0	1,0	-**	-	-	-	0.7	100
Central	Bahçeköy	1	-	-	-	-	-	-	1	100
	Paşaköy	0,0,0	0,0	0,0	-	-	-	-	0	0.00
	Körprücüler	1,1,1,1,2	0	-	-	-	-	-	0.9	75
	Çayırköy	1,1,1	0,0	1	-	-	-	-	0.6	80
BOLU	Adaköy	1,0,0,0	-	-	-	-	-	-	0.25	25
Dörtdivan	Deveciler	1,0,0,0	-	-	-	-	-	-	0.25	25
	Yayalar	0,0,1,1,1,1	-	0		-	-	-	0.5	57.14
	Kadılar	1	-	-	0	-	-	-	0.7	50
BOLU	H.Dede	1,1,1,1,0	0	1,1	-	-	-	-	0.8	100
Mudurnu	Topardıç	1,0,0,0,0,0,0,0	-	-	0	-	-	-	012	14.38
	Sırçalı	1,1,1,0,0,0,0,0,0	-	-	-	0	-	-	0.3	30
	Mean								**0.51**	**54.71**
NİĞDE	Merkez	-	0	-	0,0	-	-	-	0	0
	Gölcük	-	-	-	0,0	-	-	-	0	0
	Konaklı	-	6.52	-	0,0	-	-	-	0.88	33.33
	Alay	2	-	0		-	0	-	1.53	33.33
	Hasaköy	-	0,0	-	0,1,21.62	-	-	-	3.18	40
	Divarlı	-	0	-	0,1,0	-	-	-	0.37	25
	Mean								**0.99**	**21.94**
NEVŞEHİR	Merkez	-	16,13.8,23	-	3	-	-	-	10.65	100
Kaymaklı										
NEVŞEHİR	Merkez	0	-	-	0,2	-	-	-	0.78	33.33
Derinkuyu										
NEVŞEHİR	Mazı	-	12,23	-			-	4	7.57	100
Ürgüp										
	Mean								**6.33**	**77.77**
	Regional mean								**2.61**	**51.47**

* Disease rate of Resy variety in 5 different storage in village; There were more than one variety in the same storage.

** This variety was found in selected storage randomly to survey.

Table 2. The Disease *Colletotrichum coccodes* in tubers imported by private sector in 1993 with respect to potato varieties.

1.Company	**Granola**				**Panda**				**Hertha**				**Hermes**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
	1	10	260	A	2	4	139	A	2	5	120	A	2	3	190	C
									8	2			1	45		
									19	2			3	2		
	Ernst Tolz				**Agria**				**Planta**							
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**				
2.Company	1	47.5	20	A	3	23.5	117	A	-	-	230	A				
	1	37.5														
	Shepody				**R. Burbank**											
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**								
	-	-		1000	A	-	-	2434	A							
	Apollo				**Resy**				**Marfona**							
3.Company	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**				
	1	50	-	E	2	25	210	A	2	20	50	A				
	1	40							1	10						
	Ausonia				**Scala**				**Semena**				**Planta**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
	-	-	40	A	-	-	1	A	-	-	1	0	3	11	-	C
4.Company									-	-	67	A			1	O
															4	K
	Ausonia				**Marfona**											
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**								
	3	10	415	A	1	6.5	292.5	A								
	1	30			2	30										
5.Company	1	5														
	1	10														
	1	20														
	1	12.5														

1. Disease rate (%).
2. Amount of the lot the disease determined (t).
3. Amount of the lot disease free (t).
4. Category of the propagating material
(E: Elite, A: Basic, O: Pre, Basic, S: Certified, K: Controlled)

cause rotting of the tubers; they may shrink in long storage (Pavlista et al 1996). In also this study, the same results of surface inoculation and 4°C. But, at 22°C slightly pitted, pale brown, bronze, local dryings was occured on the tubers. Outer covering of the tubers may easily separate from the flesh at these areas. Bottom of the peel is black because of the agent. These symptoms spread towards the flesh parts causing dry rotting of the whole tuber. However, this black occurrence may be concealed by the *Fusarium* spp. or the other saprophytic fungi symptoms.

Table 3. Isolates used in variety reaction studies and dimensions of growth of *Colletotrichum coccodes* on tubers.

Variety	Mean dimension of wounds (cm)				
	I.	II.	III.	IV.	Mean
Lola	0.01*	0.65	1.20	1.02	0.78 B
Granola	0.00	0.00	0.45	1.08	0.38 A
Donella	0.91	0.01	1.00	1.10	0.75 B
Cosmos	0.95	1.00	1.10	1.25	1.07 C
Resy	0.97	0.92	0.90	1.45	1.06 C
Agata	1.48	0.82	1.13	1.10	1.13 C
Marfona	1.25	1.23	0.82	1.50	1.20 D
Fienna	1.32	1.20	1.15	1.32	1.20 D
Marabel	0.95	1.10	1.10	1.20	1.20 D
Agria	1.70	1.35	1.00	1.24	1.32 E
Mean	0.95	0.76	0.98	1.22	1.01

P: 0.05 (LSD)

* only at the periphere of disc hole

I. Afyon/Sandıklı, variety unknown

II. Bolu, Çayırköy (Çamönü), Marfona

III. Isolation from a study, Concorde

IV. From a private sector storage, Planta

Central Anatolia Region is the center of the potato production for the country. Several effective measures should be taken to prevent the spread of the disease. Although, according to the regulations related with the Seed Control and Certificate, there are several restrictions for the wilting agents (*Fusarium, Verticillium*), *C. coccodes* has not included in these lists. This pathogen should be included in such lists and additional measures should be taken. Since the agent stands alive in soil for more than 8 years (Dilard ve Cobb, 1998), chemical control studies should be started at least to decrease the inoculum level of the pathogen in soil.

ÖZET

ORTA ANADOLU BÖLGESİ'NDE PATATES DEPOLARINDA *Colletotrichum coccodes* (Wallr.) S.J. HUGHES'İN BULUNUŞ VE YAYILIŞ ORANLARI İLE ÇEŞİTLERİN ETMENE KARŞI REAKSİYONLARININ TESPİTİ

Çalışmalar 1990-1997 yılları arasında yürütülmüştür. Sürveyler Bolu, Nevşehir ve Niğde illerinde çiftçi depolarında sertifikalı tohumluk veya yemeklik patates yığınlarında ve özel şirketler tarafından ithali yapılan tohumluk patates çeşitlerinde yürütülmüştür. Hastalığın bulunuş oranı Bölge'de %2.61, yayılış oranı %51.47 olarak

tespit edilmiştir. Test edilen patates çeşitlerinin tümü hastalığa yakalanmış, çeşitlere göre yumruda ort. 0.38-1.32 cm çapında gelişme tespit edilmiştir. Yüzeysel inokulasyonlarda hastalık gelişmesi gözlenmemiştir.

Anahtar kelimeler: Patates, *Colletotrichum coccodes*, Çeşit dayanıklılığı

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J. Turk. Phytopath., Vol. 29, No. 2-3, 61-69, 2000 ISSN 0378 - 8024

Pathogenicity of the Fungi Determined on Tubers from Potato Storages in Erzurum, Türkiye

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ABSTRACT

A total 3126 potato tubers were collected from 103 potato storages located in five districts of Erzurum during 1997 and 1998. Of those, 860 tubers with symptom were used for isolation of the causal agent fungi. Twenty-five fungus species belonging to 17 genera were isolated and identified. The most prevalent fungus genera were found to be *Fusarium, Rhizoctonia, Helminthosporium, Penicillium* and *Doratomyces*. Approximately 48 % of potato tubers examined were infested with *Rhizoctonia solani*. Pathogenicity studies showed that *Fusarium sambucinum, F. solani, F. culmorum, F. oxysporum* and *Pythium ultimum* were causing severe rots in potato tubers of three different commercial potato varieties (cvs. Agria, Granula and Marfona).

Key words: Potato, Tubers, Fungal Pathogens, Pathogenicity

INTRODUCTION

Potato (*Solanum tuberosum* L.) and its products are known to be the most important source of food for human beings. An annual potato production was estimated as 425 million tons in the world. The annual yield losses of potato crop in the developing countries like Türkiye was determined to be 32.4 % because of insect, weed and diseases. Of this yield reduction, diseases were responsible of 21.8 % (Anonymous, 1993). Thus, many studies have been conducted to decrease yield losses due to diseases and increase the quality and quantity of potato production in the world.

Potato is one of the most commonly grown crop in Erzurum, Türkiye. An annual potato yield is estimated as 158 760 tons from 10 117 hectares planted in Erzurum (Anonymous, 1998). There are many pathogenic microorganisms such as fungi and bacteria which can cause economic yield losses because of severe diseases under unfavorable storage conditions. Considering most of the potato diseases are seed-borne, the importance of storages may be well-understood for managing of potato diseases in

the field. Except viruses, the diseases caused by fungi, bacteria, phytoplasmas and nematodes are responsible for 20 % yield losses of total annual potato production, and 42 % of which was due to storage diseases (Turkensteen and Eraslan, 1985).

In the previous studies, a number of pathogenic fungus species were isolated from potato tubers in the storages of Türkiye (Gündüz, 1977; Gülsoy, 1980, 1982; Turkensteen and Eraslan, 1985; Çolakoğlu, 1986, 1993; Öz et al., 1988; Demirci and Döken, 1993). Gündüz (1977), had identified 30 fungus species in 16 genera from the samples of potato and onion in the storages of Erzurum, Türkiye. The most aboundant fungus species were determined as *Penicillium* spp., *Trichothecium roseum*, *Fusarium sulphureum*, *F. equiseti* and *Ulocladium botrytis*. His pathogenicity study results showed that 62.5 % of rotted potato tubers were caused by *F. sulphureum* isolates. The data of an other study conducted in 1983 demonstrated that *F. sulphureum* and *Helminthosporium solani* the causal agents of potato dry rot and silver scurf, respectively, were two important pathogens of potatoes in the storages of Erzurum provinces (Turkensteen and Eraslan, 1985). In addition, Çolakoğlu (1986) isolated and identified 46 species of 16 fungi genera from potato and onion storages. In that study, the isolates in the genera of *Mucor, Rhizopus, Aspergillus, Penicillium, Botrytis, Fusarium* were determined at high incidence. However, the results of another work carried out by Çolakoğlu (1993) showed that the most common fungal pathogens in potato storages were *Colletotrichum, Chrysosporium* and *Fusarium*.

Recent years, that, complaining of potato growers have been increased due to yield losses caused by diseased, showed the importance of determination of the potential fungal agents from tubers which causes economically important diseases in the storages. The objectives of this study were to determine the fungi associated with potato tubers from storages, and to test the pathogenicity of the isolated fungal species on three different potato varieties commercially grown in the province of Erzurum.

MATERIALS and METHODS

During 1997-1998, diseased potato tubers were obtained from potato storages in five districts of Erzurum, including Center, Aşkale, Horasan, Ilıca and Pasinler, located in Eastern Anatolia Region of Türkiye (Table 1). At least 20 tubers were randomly picked up from each storage, and then put into labeled polyethylene bags. After all samples were washed with top water and dried in the laboratory, they were examined for presence or absence of sclerotia of *Rhizoctonia solani* on the surface of tubers. All other symptomatic potato tubers were kept in the refrigerator at 4°C for further isolation studies.

In the present study, out of 3126 potato tubers were obtained from 103 potato storages, total 860 potato tubers with different symptoms were used for isolation (Table 1).

Table 1. Locations of samples were taken from potato storages, and the number of tubers were examined and used for isolation.

Locations	Number of storages	Number of tubers examined	Number of tubers used for isolation
Aşkale	13	301	68
Horasan	7	158	33
Ilıca	10	246	55
Center	52	1538	487
Pasinler	21	883	217
Total	103	3126	860

Small pieces (0.5-1 cm in diameter) from each of the symptomatic potato tubers were excised with sterile blade, disinfected in sodium hypochlorite solution (0.5 %) for 2 min, rinsed twice in sterile distilled water, and dried with filter paper. Pieces of tubers were removed aseptically, and placed on 9 cm diameter petri plates of Potato Dextrose Agar (PDA). The plates were incubated at 25°C for 7-10 days. The isolates of fungi grown on the plates were prufied and identified based on the previously published literatures (Gerlach and Nirenberg, 1982; Hooker, 1986; Hasenekoğlu, 1991).

The pathogenicity studies were performed on three different potato varieties (cvs Agria, Granula and Marfona). Healthy potato tubers (6-8 cm in diameter) were surface disinfected in 2.5 % of sodium hypochlorite solution for 3 min, rinsed with sterile top water and then left for drying (Hide et al., 1977). The method of Seppanen (1981a) was used for inoculation with minor modifications. For this reason, a disk (4 mm in diameter) of the fungal culture grown on PDA for 10 days was placed in a wound of 6 mm in diameter and 1 cm deep on each of the same size tubers made using a cork borer. Then, the wounds were sealed with parafilm. For pathogenicity test, only one representative isolate of each fungus species was used for inoculation on three different potato tubers. Sterile PDA disks were used as control. The inoculated tubers were kept in a growth chamber (95±5 % relative humidity) at 20±2°C for 4 weeks. After incubation, the tubers were halved longitudinally through the infection center, and diameter of diseased areas was measured. Data were subjected to analysis of variance, and Duncan's multiple range test was used to determine differences among the means of species.

RESULTS

The fungal species isolated from potato tubers, and their origins and distribution were listed in Table 2. The incidence of *R. solani*, which was determined by counting the tubers with sclerotia, were 58.3 %, 41.8 %, 56.1 %, 42.6 %, 40.5 % in potato tubers in the samples taken from Aşkale, Horasan, Ilıca, Center and Pasinler, respectively.

Table 2. The number and geographical distribution of the isolates of fungi from potato storages in Erzurum provinces, Türkiye.

Fungi species	Locations					
	Aşkale	Horasan	Ilıca	Center	Pasinler	Total
Alternaria alternata	3	5	-	57	29	94
Botrytis cinerea	-	-	4	-	-	4
Colletotrichum coccodes	14	5	3	44	9	75
Curvularia harvei	-	-	-	-	3	3
Curvularia prasadii	-	-	-	3	1	4
Cylindrocarpon sp.	-	3	-	14	18	35
Doratomyces stemonitis	21	12	6	112	72	223
Fusarium acuminatum	11	-	1	113	32	157
Fusarium culmorum	-	-	-	2	1	3
Fusarium equiseti	15	14	20	117	44	210
Fusarium floccifeum	4	2	3	9	5	23
Fusarium oxysporum	11	5	3	46	31	96
Fusarium sambucinum	1	-	6	55	11	73
Fusarium solani	1	-	4	2	11	18
Fusarium verticilloides	-	-	3	-	-	3
Gliocladium catenulatum	12	3	-	87	38	140
Helminthosporium solani	15	14	26	159	46	260
Penicillium spp.	23	5	20	157	50	255
Pythium ultimum	2	-	-	1	-	3
Trichoderma spp.	-	-	-	3	1	4
Trichocladium asperum	3	-	1	13	1	18
Trichothecium roseum	-	-	-	6	-	6
Ulocladium atrum	6	5	1	34	43	89
Verticillium tenerum	-	-	-	8	-	8

In this study, total 1804 fungal isolates were obtained. Of these isolates, 32.3 % belonged to species of *Fusarium* spp. (11.6 % *F. equiseti*, 8.7 % *F. acuminatum*, 5.3 % *F. oxysporum*, 4.0 % *F. sambucinum*, 1.3 % *F. floccifeum*, 1.0 % *F. solani*, 0.2 % *F. culmorum*, 0.2 % *F. verticilloides*), 14.4 % *Helminthosporium solani*, 14.1 % *Penicillium* spp., and 12.4 % *Doratomyces stemonitis*, respectively. Furthermore, *Gliocladium catenulatum* (7.8 %), *Alternaria alternata* (5.2 %), *Ulocladium atrum* (4.9 %), *Colletotrichum coccodes* (4.2 %), *Cylindrocarpon* sp. (1.9 %), *Trichocladium asperum* (1.0 %), *Verticillium tenerum* (0.4 %), *Trichothecium roseum* (0.3 %). *Botrytis cinerea, Curvularia harvei, C. prasadii, Pythium ultimum* and *Trichoderma* spp. were also isolated at low incidence.

Pathogenicity results of the fungus species tested on three different potato varieties (cvs. Agria, Granula, Marfona) were summarized in Table 3. The data showed that the differences in both fungus species and potato varieties have significant effect on disease severity. The results showed that severe rottings were caused by *F. culmorum, F. oxysporum, F. sambucinum, F. solani, Penicillium* sp. and *P. ultimum* on tubers of Agria and Granula; by *B. cinerea* and *F. acuminatum* on Granula; and by *F. culmorum, F. sambucinum, F. solani* and *P. ultimum* on Marfona, respectively (Table 3). However, the fungi isolated from tuber surfaces and/or seconder invaders were found not to cause serious tuber rotting. No symptoms was observed on controls.

Table 3. Pathogenicity test results of the fungi species on three different potato varieties.

Fungi species	Potato varieties[x]		
	Agria	Granula	Marfona
Alternaria alternata	11.0 e[y]	9.3 de	8.0 de
Botrytis cinerea	11.3 e	30.3 b	7.7 de
Colletotrichum coccodes	8.3 e	8.0 e	8.0 de
Curvularia harvei	7.0 e	7.7 e	7.0 de
Curvularia prasadii	7.3 e	7.3 e	7.0 de
Cylindrocarpon sp.	7.3 e	7.7 e	8.3 de
Doratomyces stemonitis	7.3 e	9.0 e	7.7 de
Fusarium acuminatum	11.0 e	27.0 bc	8.7 de
Fusarium culmorum	58.0 ab	50.0 a	14.7 d
Fusarium equiseti	7.3 e	10.7 de	7.0 de
Fusarium floccifeum	12.0 e	11.0 de	8.3 de
Fusarium oxysporum	51.3 bc	52.7 a	13.3 de
Fusarium sambucinum	50.3 c	57.3 a	41.0 b
Fusarium solani	54.7 abc	54.0 a	28.7 c
Fusarium verticilloides	12.0 e	13.0 de	11.3 de
Gliocladium catenulatum	8.7 e	7.7 e	8.3 de
Helminthosporium solani	7.7 e	7.7 e	7.3 de
Penicillium sp.	21.0 d	19.0 cd	13.7 de
Pythium ultimum	59.0 a	59.3 a	57.3 a
Rhizoctonia solani	10.3 e	11.3 de	11.3 e
Trichoderma sp.	7.3 e	12.0 de	8.3 de
Trichocladium asperum	11.3 e	14.0 de	7.0 de
Trichothecium roseum	7.0 e	7.0 e	7.0 de
Ulocladium atrum	7.0 e	7.0 e	7.0 de
Verticillium tenerum	7.0 e	7.7 e	7.0 de
Control	6.0 e	6.3 e	6.0 e

[x] Means of diseased area in diameter (mm) of potato tubers tested.

[y] Means in a column followed by the same letter are not significantly different ($P<0.01$) according to Duncan's multiple range test.

DISCUSSION

In this study, *Fusarium* spp. (32.3 %) were predominantly isolated from tubers of potato storages in Erzurum. Our data confirmed the observations in the previous studies which showed that *Fusarium* spp. were frequently isolated from potato tubers and caused yield losses in potato storages of Sakarya and Bolu in Türkiye (Gülsoy, 1980, 1982). Dry rot among the potato tuber rot diseases caused by *Fusarium* spp. was also reported to be the most common problem in potato storages in Türkiye (Turkensteen and Eraslan, 1985). *F. sambucinum* and *F. solani* var. *coeruleum* were determined as the most common causal agent of dry rot of potato tubers in North America (Schisler et al.,1995). Confirming this evidence, the pathogenicity data showed that *F. oxysporum*, *F. solani*, *F. sambucinum* and *F. culmorum* were more aggressive pathogens than other fungal agents isolated in this work (Table 3). *F. sambucinum* is also well known causal agent of dry rot disease in Germany, Poland, Canada, England and USA (Boyd and Tickle, 1972; Jeffries et al., 1984). Our pathogenicity studies demonstrated *F. sambucinum* (4 % of the isolates) was quite virulent pathogens on all the potato varieties tested. This result supported the findings of some other previous studies in which *F. oxysporum*, *F. solani* and *F. sambucinum* were determined as important causal agent of dry rot of potato in Türkiye (Gündüz, 1977; Gülsoy, 1980, 1982; Öz et al., 1988). The data showed that *F. culmorum* was another important pathogen causing severe dry rot on all three potato varieties. This is the evidence proving the observation of Seppanen (1981b), but conflicting with the results of Gülsoy (1980, 1982).

All the potato tubers were examined in this study, 48 % of those were found to be infested with sclerotia of *R. solani*. The average number of potato tubers diseased by *R. solani* in potato storages of Erzurum were determined as 35.8 % and 28.4 % in 1989 and 1990, respectively (Demirci and Döken, 1993). On the other hand, Çolakoğlu (1986, 1993) conducted two different studies to determine the distribution of mycoflora in potato and onions storages in Erzurum could not find *R. solani* at all. Therefore, the data showed increasing incidence of *R. solani* during last decade. All of the isolates of *R. solani* and another agent identified as *C. coccodes* isolated from the surface of potato tubers did not induce symptoms on tubers of potato varieties tested. This result may implied that even through infestation of storaged potato tubers with these agents (*R. solani* and *C. coccodes*) may not cause yield losses in potato storages, but if they are introduced from storage to field by using the infested tubers as seed, it may be responsible of severe damage in the field. Since, the fungi species, *A. alternata*, *C. coccodes*, *F. acuminatum*, *F. equiseti*, *F. solani*, *R. solani* and *U. atrum* isolated from stored potato tubers were well-known primary pathogen of potato causing disease on roots, stems and leaves of potato plants in field condition, of Erzurum, Türkiye (Demirci and Döken, 1989).

An other important rotting pathogen of potato tubers was *P. ultimum* isolated at low incidence in this study. Similar results were obtained in some other previous studies, too (Gülsoy, 1980,1982; Öz et al., 1988). *B. cinerea* and *Penicillium* sp. were determined two other tuber rotting pathogenic fungi. All of the other fungi isolated in this study did not induce any visible symptoms on the tubers of all three varieties. This finding indicate that these fungi isolates were not pathogenic on potato tubers. Perhaps they may colonized on rotted potato tubers as either saprophytic or epiphytic organism. This interpretation is corroborating with the observations of many other researchers who reported these fungi were nonpathogenic, except Gündüz (1977) claimed *Doratomyces purpureotuscus* was minor pathogen of potato tubers.

Our studies showed that improper storage conditions provide favorable environment for pathogenic fungi which cause economically important yield losses in potato production in the storages. The contaminated seeds are also considered the major source of primary inoculum as well as the major means of long-distance spread of these pathogenic fungi in the field. Thus, the effective management strategies for elimination of yield losses in potato stocks require storage of clean, healthy and pathogen-free potato tubers as well as improvement in storage conditions.

ÖZET

ERZURUM İLİ PATATES DEPOLARINDAKİ YUMRULARDA SAPTANAN FUNGUSLARIN PATOJENİTELERİ

Erzurum ilinde 1997-1998 yıllarında yapılan bu çalışmada, 5 ilçedeki 103 patates deposundan 3126 patates yumrusu alınmış ve simptom gözlenen 860 adet yumrudan izolasyon yapılmıştır. Çalışma sonucunda, 17 fungus cinsi ve bu cinslere ait 25 tür izole edilmiştir. Fungus cinslerinden en yaygın olanları *Fusarium, Rhizoctonia, Helminthosporium, Penicillium* ve *Doratomyces* olarak saptanmıştır. İncelenen tüm yumruların *Rhizoctonia solani* ile bulaşıklık oranının % 48 olduğu belirlenmiştir. Patojenite çalışmalarında *Fusarium sambucinum, F. solani, F. culmorum, F. oxysporum* ve *Pythium ultimum*'un 3 patates çeşidinde (Agria, Granula ve Marfona) de önemli derecede çürümeler meydana getirdiği saptanmıştır.

Anahtar kelimeler: Patates, Yumru, Fungal patojenler, Patojenite

ACKNOWLEDGMENT

We thank Dr. H. NIRENBERG (Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Mikrobiologie, Berlin, Germany), Dr. Dilek AZAZ (Department of Biology, Faculty of Science, University of Balıkesir, Turkey) and Dr.

Gürsel HATAT (Department of Plant Protection, Faculty of Agriculture, University of Ondokuz Mayıs, Samsun, Turkey) for identification of some fungus isolates.

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J. Turk. Phytopath., Vol. 29, No. 2-3, 71-77, 2000 ISSN 0378 - 8024

Isolation and Bioassay of *Phoma tracheiphila* (Petri) Kanc. et Ghik. Toxin

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ABSTRACT

Partially purified toxin of *Phoma tracheiphila* (Petri) Kanc. et Ghik. was assayed and standardizated to select resistant line or lines to the toxin through tissue culture. Disease severities of the toxin for each dilution were different. However ED_{50} was almost 1/50 dilution, on both scale, except *Lycopersicon esculentum* ES-58 which was the most sensitive variant in the experiment. Disease severity caused toxin on the seedlings was declined significantly below 1/40 toxin dilution. There were observed no difference on disease severity of caused between 1/60-1/100 serially diluted toxin on citrus but appeared variation on tomato. As a result, non-host spesific toxin of the disease agent was very useful to investigate the correlation between ED_{50} and host reaction.

Key words: *Phoma tracheiphila*, Toxin, Isolation, Bioassay

INTRODUCTION

Mal secco caused by *Phoma tracheiphila* (Petri) Kanc. et Ghik. is one of the most serious vascular fungal disease of lemon in Mediterranean Region (Solel and Oren, 1975; Solel and Salerno, 1988). So far, the disease that is important strikingly in Turkey was reported by Istranca (1952) in Adana in 1940. Phytotoxin that has role in pathogenesis produced by *P. tracheiphila* is non-host spesific and contains 29.5 % carbonhydrate and 36 % peptide (Nachmias et al., 1977; 1978; 1980). The application of the culture filtrate of *P. tracheiphila* to citrus seedlings have shown that has relatively been possible to classify the varieties as resistant or susceptible (Akteke, 1979).

Effective dose of any toxin bath should be standardizated on seedlings to maintaince uniform selection pressure for *in vitro* selection investigations. In order to use as a stress factor mal secco toxin for *in vitro* lemon calli selection, it should be calibrated by the seedlings bioassay. However calibration of the toxin takes long time if

the calli are used as a plant material. The present investigation reports the isolation, bioassay and calibration of mal seccin by using seedlings and shoot tips of two different test plants.

MATERIALS and METHODS

Toxin Production and Isolation

P. tracheiphila was provided kindly from Dr. İsmail Çimen (Alata Horticultural Research Institute, İçel-Turkey) and maintained on PDA culture medium. Liquid cultures of the pathogen for phytotoxin production was prepared according to Nachmias et al., (1977). Five fungal discs (4 mm) were transferred into steril, dark bottles (1 l) containing 100 ml of culture medium (2.0 g aspargine, 1.5 g KH_2PO_4, 1.0 g $MgSO_4.7H_2O$, 20.0 mg $ZnSO_4.7H_2O$, 10.0 mg $FeCl_3$, 10.0 mg Thiamine HCl, 5.0 mg Pyridoxine, 20.0 g glycose and 1 l distilled water). Bottles were incubated in a climate room in dark at 27 ± 1°C for 30 days.

Toxin was purified partially according to Nadel and Spiegel-Roy (1987). Culture liquids were filtered through glass wool and 2 layers of Watmann 3 MM paper to remove the fungal mat. The filtrate was then concentrated to 5 ml at 45°C by rotary evaporator and lyophilized following precipitation with 3 vol. of acetone at -18°C overnight. After the gummy raw toxin was dissolved in sodium phosphate buffer (1/1.5 w/v; pH 5.5), pellet was removed by centrifugation (Beckman JA-20 rotor) at 15.000 rpm. and 4°C for 10 min. Supernatant including partially purified toxin was filtered through 22 µm milipore filter and stored at deep freeze.

Calibration of Toxin

Tomato seedlings, shoot tips of lemon (*Citrus lemon* (L) Burm. f. cv Kütdiken (KT) and sour orange (*Citrus aurantium* L.) (SO) were used for calibration.

Standardization were carried out by dilution of raw toxin with water in ratios of 1/10, 1/20, 1/30, 1/40, 1/50, 1/60, 1/70, 1/80, 1/90 and 1/100. Tomato seedlings (10-15 days old) were placed in 1.5 ml eppendorf tubes containing diluted toxin and incubated in a growth chamber at high humidity and 27 ± 1°C. Controls contained water only. First visible symptoms were observed in 5-6 hours after inoculation and scored for severity after 20 hours. Disease severity was calculated on original 0-8 scale (0=no symptom, 1=slight wilted leaves, 2=slight curved stem, slight wilted leaves, 3=slight curved stem, mild wilted leaves, 4=moderate wilted stem, slight wilted leaves, 5=moderate curved stem, mild wilted leaves with curled tips, 6=severe curved stem with collapsed leaves, 7=collapsed stem with dead leaves, 8=whole dead plant).

Fresh and unique shoot tips with 4-5 leaves of lemon and sour orange were incubated with diluted toxin and maintained as described above. First visible symptoms were observed 4 days after inoculation and scored for symptom severity. Scoring developed by us was based on a scale of 0-4 (0=no symptom, 1=slight wilted, very few necrotic area, 2=moderate wilted intensive necrotics area, 3=scalded leaves with severe wilted, expanded necrotic areas, 4=total tissue collaps, coalesced necrotic lesions).

Data Analysis

The average score was designated as the disease index (Karman, 1971). The ED_{50} value for each species, defined as the concentration of the toxin causing 50 % disease of the population was determined using multiple regression analyse.

RESULTS and DISCUSSION

As shown in Table 1 the ratio of 1/10 toxin dilution caused high mortality on all species. The toxin did not produce symptoms of identical severity among 3 tomato cultivars. The results may be attributed to their genetic differences. Nadel and Sahar (1983) reported that cultivars of the same species, intra spesific differences in sensitivity to the mal seccin varied to range from very susceptible to very tolerant, for cucumber and mung bean and the spesific cultivar may not also present in all species of the cultivars. Intra spesific variants of the citrus gave more uniform reaction to determine effective dose value. The uniformity of the reaction may be considered that citrus is original host of the fungi. In any bioassay uniformity of response is also related to genetic uniformity of test plants (Nadel and Sahar, 1983). Effect of low dosages between 1/50 and 1/100 toxin dilutions was similar on lemon and sour orange. Disease severity caused by 1/20, 1/30 and 1/40 toxin dilutions changed between 75-100 % (Table 2) and the response in virulence by 1/10, 1/20, 1/30 and 1/40 toxin dilutions to tomato seedlings were similar to that of citrus shoot tips but occasionally variations on tomato. The ED_{50} value for each species was 1/50, 1/55, 1/70, 1/50 and 1/50 by regression analysis for *L. esculentum* 9099, H-2274, ES-58 which was the most sensitive variant, lemon and sour orange, respectively (Table 2). Disease severity decreased strikingly below 1/40 toxin dilution and reduction to 1/50 from 1/40 toxin dilution was to 37.5 %, 46 %, 65 %, 55 %, 35 % from 77.5 %, 75 %, 86.25 %, 75 %, 75 % for each species (tomato (9099), (H-2274), (ES-58), lemon and sour orange, respectively) (Table 2).

Although Nadel and Sahar (1983) reported that lemon was not a good test plant for bioassay, we have obtained acceptable results with it. The symptoms on lemon leaves produced by the toxin (Figure 1) were identical to the results of Nachmias et al., (1977)

Table 1. Scale of symptoms for calibration of toxin observed on tomato seedlings and citrus shoot tips.

Plants	Number of plants tested for each toxin dilution	Toxin dilutions										
		1/10	1/20	1/30	1/40	1/50	1/60	1/70	1/80	1/90	1/100	Control
		Disease Index										
Tomato (*L. esculentum* 9099)	10	8*	7	6.2	6.2	3	3.1	1.4	1	1.4	2.3	0
Tomato Hybrid (*L. esculentum* H-2274)	10	8	8	7.5	6	3.7	3.8	3.8	2.2	2.1	1.9	0
Tomato Hybrid (*L. esculentum* ES-58)	10	8	8	7	6.9	5.2	5	4.6	3.4	4	3.7	0
Limon (Kütdiken) (*Citrus lemon* Burm f.)	5	4*	4	3	3	2.2	1	1	1	1	1	0
Sour Orange (*Citrus aurantium* L.)	5	4	3	3.2	3	1.4	1	1	1	1	1	0

* Disease index was scored between 0-8 for tomato seedlings and 0-4 for citrus as described in the text.

Table 2. Disease severity of toxin on tomato seedlings and citrus shoot tips.

Ratio of Toxin Dilution	Tomato			Citrus	
	9099	H-2274	ES-58	Kütdiken	Sour Orange
	Disease Severity (%)				
1/10	100	100	100	100	100
1/20	87.5	100	100	100	75
1/30	77.5	94	87.5	75	80
1/40	77.5	75	86.25	75	75
1/50	37.5	46	65	55	35
1/60	38.75	47.5	62.5	25	25
1/70	17.5	47.5	57.5	25	25
1/80	12.5	27.5	42.5	25	25
1/90	17.5	26.25	50	25	25
1/100	28.75	26.25	46.25	25	25
Control	0	0	0	0	0

who obtained results which the injection of 400 mg pure toxin into lemon seedling leaves caused local necrosis within 12 hours followed by leaf drying. The results suggested that non-host spesific toxin of mal secco may be used to investigate relations

of ED_{50}-host reaction. The toxin has very vast host range including avacado, cucumber and tomato (Nachmias et al., 1977). Although bioassays conducted on different cultivars of the same species gave widely different results, ED_{50} was identical.

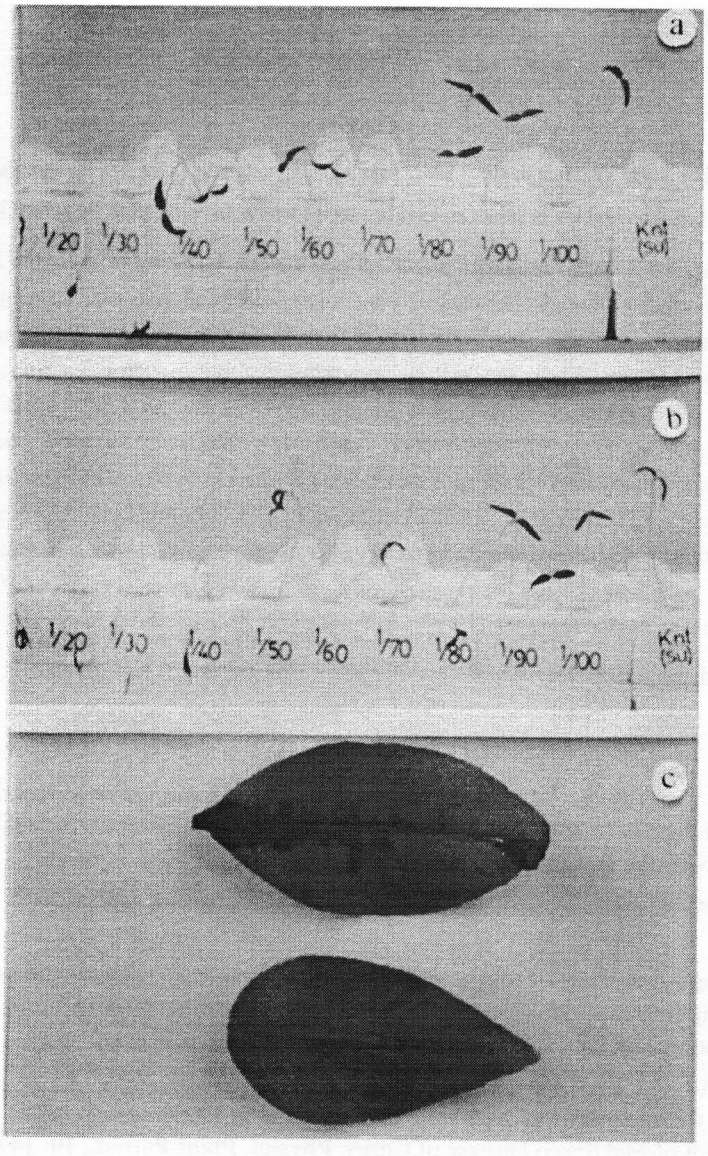

Figure 1. Symptoms of Mal Secco toxin on tomato seedlings and lemon leaves a) first visible symptoms in 6 hours after treatment of toxin on *Lycopersicon esculentum* H-2274, b) the symptoms in 20 hours after treatment of toxin, c) observed necrosis caused by mal secco toxin on lemon leaves, treated (above) and control (below).

ACKNOWLEDGMENTS

We wish to thank Dr. Ali ERKILIÇ for assistance in statistical analysis. We are also grateful to Scientific and Technical Research Council of Turkey (TÜBİTAK-NATO A2) that supported partly the project as a part of doctoral thesis (Çukurova University 2000).

ÖZET

PHOMA TRACHEIPHILA (PETRİ) KANC. ET GHİK. TOKSİNİNİN İZOLASYONU VE BİYOLOJİK AKTİVİTESİNİN TESTLENMESİ

Phoma tracheiphila'nın kısmi olarak saflaştırılan toksini biyolojik aktivitesi için bitkiler üzerinde test edildi ve doku kültürü teknik yöntemleriyle etmenin toksinine dayanıklı hat veya hatların seleksiyonu amacıyla standardize edildi. Denemede kullanılan tüm bitkiler için toksinin farklı sulandırma düzeylerinde bitkiyi hastalandırma şiddeti birbirinden farklı olmuştur. Ancak ED_{50} etkisi denemedeki en duyarlı olan *Lycopersicon esculentum* ES-58 çeşidi hariç tüm bitkiler için her iki skala üzerinde hemen hemen 1/50 olmuştur. Hastalık şiddeti 1/40'ın altındaki sulandırma oranları için önemli bir azalma göstermiştir. 1/60 - 1/100 arası sulandırılmış toksinin neden olduğu hastalık şiddetleri arasında turunçgiller üzerinde farklılık gözlenmemesine rağmen bu durum domates üzerinde biraz değişkenlik göstermiştir. Sonuç olarak Uçkurutan Hastalığının konukçuya özgü olmayan toksini, ED_{50}-konukçu reaksiyon araştırmalarında oldukça yararlı sonuçlar vermiştir.

Anahtar kelimeler: *Phoma tracheiphila*, Toksin, İzolasyon, Biyolojik Aktivite

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J. Turk. Phytopath., Vol. 29, No. 2-3, 79-84, 2000 ISSN 0378 - 8024

Fungi Isolated from Corn Kernels in the Eastern Black Sea Region

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ABSTRACT

Totally, 165 samples of corn kernels received to determinate the seed borne fungi were collected from five provinces in the Eastern Black Sea Region throughout the major corn producing areas of Turkey. A total of 26 fungal species belonging to 19 genera were isolated from corn kernels, using agar plate technique. The most common and frequently occurring fungi were *Aspergillus* spp., *Fusarium graminearum, F. proliferatum, F. verticilloides, Nigrospora oryzae, Penicillium* spp. and *Rhizopus stolonifer*. The other fungi detected to varying degrees were *Acremonium strictum, Alternaria alternata, Botrytis cinerea, Chaetomium* spp., *Cladosporium* spp., *Cochliobolus carbonus, Fusarium equiseti, F. incarnatum, F. oxysporum, Geomyces pannorum, Harzia acremonioides, Mucor* spp., *Phoma glomerata, Rhizoctonia solani, R. zeae,* binucleate *Rhizoctonia* sp., *Trichoderma* spp., *Trichothecium roseum* and *Ulocladium atrum*. Of these fungi species, *A. strictum, F. incarnatum, G. pannorum, P. glomerata* and *U. atrum* were reported for the first time on corn kernel in Turkey.

Key words: *Zea mays*, Kernels, Fungal Flora, Eastern Black Sea Region, Turkey

INTRODUCTION

Corn (maize, *Zea mays* L.), along with barley and wheat, are the three most important cereal crops in Turkey. It is also widely cultivated as cereal crop in the Eastern Black Sea Region of Turkey. This region has about 101,879 hectares growing area of corn, and total production of 174,698 tons in 1996 (Anonymous,1998).

Many fungal pathogens can be seed transmitted and by this means they can efficiently overwinter and spread. Corn is susceptible to a number of kernel rots, some of them are widely distributed (Shurtleff, 1980). These rots cause considerable damage in humid areas, especially when rainfall is above normal from silking to harvest. The climatic conditions are favorable for fungal colonization of corn kernels in the Eastern Black Sea Region.

Several fungi on corn kernels have been reported in Turkey so far (Baydar, 1978; Soran and Asan, 1987; Aktaş et al., 1998; Benlioğlu and Yıldız, 1998; Demirci and Kordali, 1999), but the incidence of each fungi in kernel samples has not been documented in detail in the Eastern Black Sea Region. The effect of kernel fungi on germination and vigour remains an important consideration in disease control programs, but seed quality has become important with the use of corn kernel for human and farm animals consumption. The objective of this study was to determine the mycoflora of corn kernel from the Eastern Black Sea Region.

MATERIALS and METHODS

Corn samples: In 1996, 165 samples of corn kernels were obtained from five provinces throughout the major corn producing areas in the Eastern Black Sea Region of Turkey: Artvin (41 samples), Rize (28 samples), Trabzon (32 samples), Giresun (30 samples) and Ordu (34 samples). Samples of corn kernels were stored in polyethylene bags at 5°C until examined.

Isolation and identification of fungi: A subsample of kernels (approximately 100 g) from each sample was surface-disinfected in 3.5% NaOCl for 1 min. Kernels were washed in three changes of sterile water, and 100 kernels per subsample (a total of 16,500 kernels) were placed in a circle 1 cm from the edge of 9 cm in diameter sterile petri plates (5 kernels per plate) containing 15 ml of potato dextrose agar (PDA). After 5 to 7 days of incubation at 25°C in the dark, the presence of fungi was assessed for all species except those within the genus *Fusarium*, which were observed after 14 days. The identification of the *Fusarium* species was made according to the taxonomic system of Gerlach and Nirenberg (1982). Identification of *Fusarium* species was also verified by Dr. H. Nirenberg (Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Mikrobiologie, Berlin-Dahlem). Determination of the remaining fungi was carried out using standard taxonomic schemes (Simmons, 1967; Ellis, 1971; Ellis, 1976, Domsch et al., 1980; Hasenekoğlu, 1991; Sneh et al., 1991). Percentage of kernel infested with fungi was estimated by microscopic and visual inspection of 100 random kernels from each sample.

RESULTS and DISCUSSION

Totally, 26 fungal species belonging to 19 genera were isolated from corn kernels in the Eastern Black Sea Region of Turkey in 1996 (Table 1). The fungi isolated in more than 5% of kernels at least from one province and the total incidence of isolation were *Aspergillus* spp. (5.07%), *Fusarium graminearum* (7.92%), *F. proliferatum* (8.33%), *F. verticilloides* (14.98%), *Nigrospora oryzae* (10.34%), *Penicillium* spp. (14.89%) and

Table 1. Incidence of fungi species in or on corn kernels from the Eastern Black Sea Region of Turkey in 1996.

Fungi	Provinces											
	Artvin (41)[a]		Rize (28)		Trabzon (32)		Giresun (30)		Ordu (34)		Total (165)	
	IS[b]	IK[c]	IS	IK	IS	IK	IS	IK	IS	IK	IS	IK
Acremonium strictum	26.83	2.34	71.43	3.71	28.13	1.56	16.67	1.00	26.47	1.18	32.73	1.94
Alternaria alternata	7.32	0.15	-[d]	-	9.38	0.38	6.67	0.33	5.88	0.12	6.06	0.19
Aspergillus spp.	43.90	5.95	92.86	11.00	34.38	0.88	46.67	5.93	47.06	2.29	51.52	5.07
Botrytis cinerea	4.88	0.34	-	-	-	-	-	-	-	-	1.21	0.09
Chaetomium spp.	12.19	0.34	-	-	9.38	0.31	-	-	8.82	0.24	6.67	0.19
Cladosporium spp.	4.88	0.10	-	-	-	-	-	-	-	-	1.21	0.02
Cochliobolus carbonus	2.44	0.15	-	-	6.25	0.38	3.33	0.20	5.88	0.18	3.64	0.18
Fusarium equiseti	9.76	0.44	-	-	-	-	-	-	-	-	2.42	0.11
Fusarium graminearum	53.66	8.24	10.71	0.21	46.88	3.56	63.33	15.47	79.41	11.29	52.12	7.92
Fusarium incarnatum	12.19	0.34	-	-	-	-	-	-	17.65	1.12	6.67	0.32
Fusarium oxysporum	-	-	7.14	0.64	-	-	-	-	-	-	1.21	0.11
Fusarium proliferatum	73.17	10.88	89.29	7.21	100	10.25	60.00	2.27	79.41	9.71	80.00	8.33
Fusarium verticilloides	85.37	26.34	64.29	6.86	75.00	16.38	26.67	1.80	73.53	18.29	66.67	14.98
Geomyces pannorum	21.95	1.56	-	-	-	-	-	-	-	-	5.46	0.39
Harzia acremonioides	-	-	14.29	1.00	-	-	6.67	0.67	-	-	3.64	0.29
Mucor spp.	39.02	4.20	7.14	0.21	9.38	0.25	13.33	0.40	20.59	0.71	19.39	1.35
Nigrospora oryzae	53.66	9.61	35.71	2.64	71.88	8.81	40.00	4.40	94.12	24.24	60.00	10.34
Penicillium spp.	95.12	13.02	85.71	11.57	90.63	12.19	86.67	23.40	91.18	14.88	90.30	14.89
Phoma glomerata	4.88	0.15	-	-	-	-	-	-	-	-	1.21	0.04
Rhizoctonia solani	4.88	0.20	-	-	-	-	-	-	5.88	0.35	2.42	0.12
Rhizoctonia zeae	-	-	-	-	9.38	0.75	3.33	0.40	-	-	2.42	0.22
Binucleate *Rhizoctonia* sp.	9.76	0.59	-	-	-	-	-	-	-	-	3.03	0.17
Rhizopus stolonifer	95.12	16.88	100	14.86	100	12.81	100	13.47	100	12.29	98.79	14.18
Trichoderma spp.	34.15	1.32	14.29	0.71	25.00	1.06	6.67	0.53	20.59	0.94	21.21	0.95
Trichothecium roseum	12.19	0.29	-	-	18.75	0.44	-	-	11.77	0.29	9.09	0.22
Ulocladium atrum	-	-	3.57	0.14	-	-	-	-	-	-	0.61	0.02

[a]( ): Number of samples, [b]IS: Percentage infested samples, [c]IK: Percentage infested kernels, [d]-: Not detected.

Rhizopus stolonifer (14.18%). The other fungi detected to varying degrees were *Acremonium strictum, Alternaria alternata, Botrytis cinerea, Chaetomium* spp., *Cladosporium* spp., *Cochliobolus carbonus, Fusarium equiseti, F. incarnatum, F. oxysporum, Geomyces pannorum, Harzia acremonioides, Mucor spp., Phoma glomerata, Rhizoctonia solani, R. zeae,* binucleate *Rhizoctonia* sp., *Trichoderma* spp., *Trichothecium roseum* and *Ulocladium atrum*. The incidence of infested kernels was detected to varying degrees but symptoms of infection were not evident. In the studies carried out previously by different researchers in Turkey, various fungi were recovered from corn kernels (Baydar, 1978; Soran and Asan, 1987; Aktaş et al., 1998; Benlioğlu and Yıldız, 1998; Demirci and Kordali, 1999). This is also first report of the isolation of *A. strictum, F. incarnatum, G. pannorum, P. glomerata* and *U. atrum* from corn kernel in Turkey.

The percentage of kernel infested with each fungus varied in among province. Only *A. strictum, Aspergillus* spp., *F. graminearum, F. proliferatum, F. verticilloides, Mucor* spp., *N. oryzae, Penicillium* spp., *R. stolonifer* and *Trichoderma* spp. were present on kernel samples from all five provinces. The predominant species of fungi were as fallows: *F. verticilloides* (26.34%), *R. stolonifer* (16.88%) and *Penicillium* spp. (13.02%) in Artvin; *R. stolonifer* (14.86%), *Penicillium* spp. (11.57%) and *Aspergillus* spp. (11.00%) in Rize; *F. verticilloides* (16.38%), *R. stolonifer* (12.81%) and *Penicillium* spp. (12.19%) in Trabzon; *Penicillium* spp. (23.40%), *F. graminearum* (15.47%) and *R. stolonifer* (13.47%) in Giresun; *N. oryzae* (24.24%), *F. verticilloides* (18.29%) and *Penicillium* spp. (14.88%) in Ordu.

Kernel infections by the fungi reduce the quality and value of seed. The most of fungi determined in this study were also associated with seed rots, seedling blights, stalk and leaf infections on corn (Shurtleff, 1980). *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. are frequently isolated from corn kernels and produce toxic mycotoxins (Shurtleff, 1980). The contamination of food and feed with mycotoxins has been associated with outbreaks of human and animal mycotoxicoses, respectively.

ÖZET

DOĞU KARADENİZ BÖLGESİ'NDE MISIR DANELERİNDEN İZOLE EDİLEN FUNGUSLAR

Türkiye'de önemli mısır üretim alanlarından biri olan Doğu Karadeniz Bölgesi'ndeki beş ilden alınan 165 örnekte, tohum kaynaklı funguslar belirlenmiştir. Agar petri tekniği kullanılarak yapılan izolasyonlarda, mısır danelerinden 19 cinse ait 26 fungus türü izole edilmiştir. Çok yaygın ve sıklıkla izole edilen fungus türleri *Aspergillus* spp., *Fusarium graminearum, F. proliferatum, F. verticilloides, Nigrospora oryzae, Penicillium* spp. ve *Rhizopus stolonifer*'dir. İzole edilen diğer türler ise *Acremonium*

strictum, Alternaria alternata, Botrytis cinerea, Chaetomium spp., *Cladosporium* spp., *Cochliobolus carbonus, Fusarium equiseti, F. incarnatum, F. oxysporum, Geomyces pannorum, Harzia acremonioides, Mucor* spp., *Phoma glomerata, Rhizoctonia solani, R. zeae*, iki nukleuslu *Rhizoctonia* sp., *Trichoderma* spp., *Trichothecium roseum* ve *Ulocladium atrum*'dur. Bu türlerden *A. strictum, F. incarnatum, G. pannorum, P. glomerata* ve *U. atrum* Türkiye'de mısır daneleri üzerinde ilk kez saptanmıştır.

Anahtar kelimeler: Mısır, Dane, Fungal Flora, Doğu Karadeniz Bölgesi, Türkiye

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J. Turk. Phytopath., Vol. 29, No. 2-3, 85-94, 2000 ISSN 0378 - 8024

Determination of Incidence of Grapevine Leafroll Associated Viruses in Some Grapevine Varieties Grown in Thrace Region•

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ABSTRACT

A survey was carried out to determine incidence of grapevine leafroll associated viruses in some grape varieties grown in Thrace region, Turkey, between 1997 and 1998. Totally 421 samples were tested for infection of GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-7 by ELISA. GLRaVs were present on 268 out of 421 vines tested individually. The highest infection ratio was by GLRaV-1 (37,05 %), followed by GLRaV-3 (33,01 %), GLRaV-2 (7.83 %) and GLRaV-7 (4.03 %), respectively. As a result of ELISA, 191 individual plants were found infected at least by 1 virus, 68 by 2 virus, 9 by 3 virus serotypes. However, no mixed infection together by 4 virus serotypes was determined. The most common mixed infection type was found by GLRaV-1 and GLRaV-3.

Key words: Grapevine, Grapevine leafroll disease, Grapevine leafroll associated viruses, Thrace Region

INTRODUCTION

Grapevine leafroll associated viruses (GLRaVs), the causal agents of one of the most important virus diseases, can decrease the quality and the quantity of the grapes in viticultural areas throughout the world (Caudwell, 1964; Bovey et al., 1980; Martelli, 1993a; Martelli et al., 1997). The disease has typical symptoms as reddening or yellowing of the leaves accompanied by rolling down of the blades (Fig.1) in addition to reduced growth together with reduced yield, smaller, greenish or whitish grapes and causes reducing the sugar content of the grapes (Baldacci et al., 1976; Monette et al., 1989; Teliz et al., 1987; Martelli, 1991; 1993; Woodham and Krake, 1983; Jordan et al., 1993; Rowhani et al., 1997). Leafroll affects red-berried or white-berried *Vitis vinifera*

• This study was carried out between 1997 and 1998 as a part of PhD thesis and was supported by University of Çukurova Research Fund

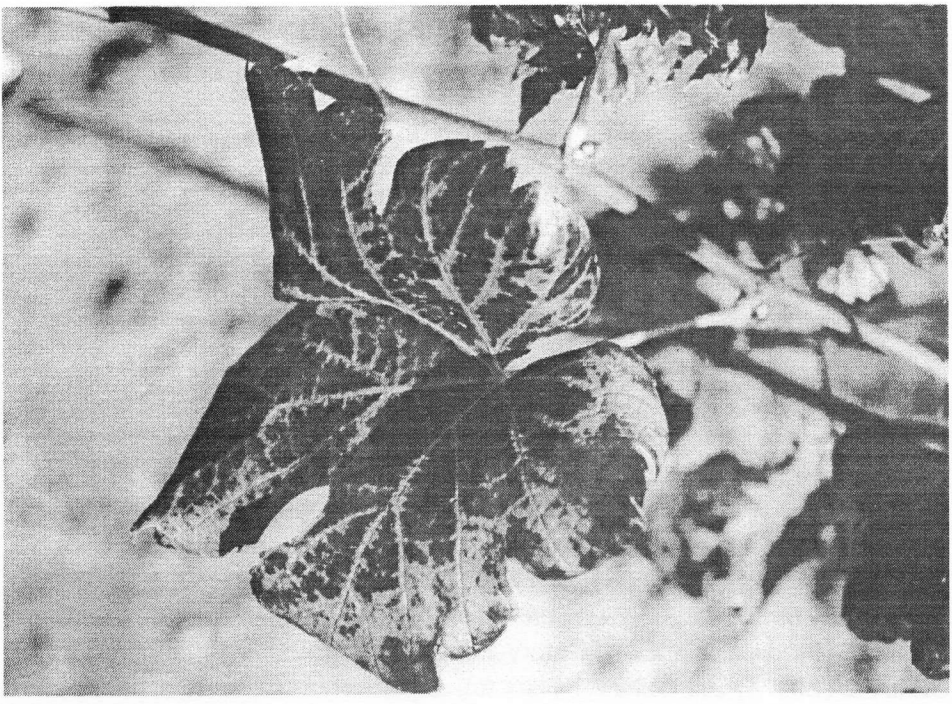

Figure 1. Reddening the leaf accompanied by rolling down of the blade on GLRaVs infected *Vitis vinifera* cv. Cinsaut (T. Cin2) in Tekirdağ.

varieties to some extent (Bovey et al., 1980, Teliz et al., 1987; Martelli, 1993; Boscia et al., 1995), whereas the American rootstock has latency, except *Vitis riparia* Gloire (Vuittenez, 1984).

Grapevine leafroll associated closterovirus particles, GLRaV-1 (Gugerli et al., 1984), GLRaV-2 (Gugerli et al., 1984), GLRaV-3 (Rosciglione and Gugerli, 1986), GLRaV-4 (Hu et al., 1990), GLRaV-5 (Walter and Zimmermann, 1990), GLRaV-6 (Boscia et al., 1995) and GLRaV-7 (Choueiri et al., 1996), can be detected in infected plants by monoclonal and policlonal antisera from leaf or cortical tissues by DAS-ELISA and Biotin\endash Sreptavidin ELISA and additionally by electron microscopy in partly purified or purified extracts (Gugerli et al., 1984; Gugerli, 1987; Zimmermann et al., 1990; Hu et al., 1990; Boscia et al., 1990a, 1995; Choueiri et al., 1996). Grapevine leafroll associated viruses (GLRaVs) can not be easily detected in *Vitis rupestris* and other American hybrids, however, ELISA and Western blot techniques gave more reliable results comparing electron microscopy (Boscia et al., 1990a, 1990b). A modification of ELISA, in which F(ab')2 antibody fragments were used, can detect grapevine leafroll associated viruses infections in infected tissues (Rowhani, 1992; Salati et al., 1993).

GLRaV-1 and GLRaV-3 were found as the most common viruses in the world and GLRaV-1 more common in some areas, however GLRAV-3 in the others (Teliz et al., 1987; Hu et al., 1990). According to the result of the studies which carried out in many viticultural regions and many varieties, GLRaV-3 were reported as the most widespread virus (Segura et al., 1993; Fortusini et al., 1993; Walter and Zimmermann, 1990; Martelli, 1993; Monette et al., 1989). In a study carried out in Lombard region, Italy, GLRaV-3 was widespread in Valtellina, but GLRaV-3 was more common in Oltrepo Pavese (Fortusini et al., 1993). In another study which has been done in Palestine, GLRaV-1 was found as the most widespread leafroll associated virus followed by GLRaV-3, GLRaV-2 and GLRaV-7 (Al-Kowni et al., 1997). Although, it was determined that GLRaV-1 infection was so common, GLRaV-3 was less in incidence in Germany (Kassemeyer, 1990). The infection ratio was found as 40 % for GLRaV-1, 21 % for GLRaV-3 and 6 % for GLRaV-7 in Turkey, respectively (Yılmaz et al., 1997). In the studies carried out in Guadiana basin and Galicia, GLRaV-3 found more widespread than GLRaV-1 (Segura et al., 1993; Fresno et al., 1997). GLRaV-3 found as the most common (77 %), followed by GLRaV-2 (27 %) and by GLRaV-1 (18 %) in Tunisia (Boulila et al., 1990).

This study was carried out to determine incidence of grapevine leafroll associated viruses in some grapevine varieties grown in Thrace region, Turkey, between 1997 and 1998.

MATERIALS and METHODS

Survey studies were carried out to determine grapevine leafroll associated virus infections in 3 important grapevine growing districts of Thrace region between 1997 and 1998. The surveys were made by collecting 3-4 samples each 25-30 cm in length randomly from individual 8 to 35 years old grapevines belong to 6 grape varieties in September and October. The collected samples were kept in labeled polyethylene bags at 4°C until brought to Virology Laboratory in Çukurova University, Faculty of Agriculture, Dept. of Plant Protection. Cortical tissues of matured canes were used for ELISA to detect the viruses. DAS-ELISA was made as described by Clark and Adams (1977) for GLRaV-3 and Direct Biotin-Avidin ELISA as described by Zrein et al. (1986) for GLRaV-1, GLRaV-2 and GLRaV-7. Antisera were provided by Bari University (GLRaV-1, GLRaV-2 and GLRaV-7), additionally GLRaV-3 was produced as described by Ball et al. (1988) and labeled with alkaline phosphatase as described by Avrameas et al. (1969) and optimum dilutions were determined as described by Clark and Bar-Joseph (1984) and used in the survey study. Optical density (A_{405}) was measured in Medispec ESR 200 ELISA plate reader.

RESULTS and DISCUSSION

Totally 421 individual samples were tested for GLRaV-1, -2, -3 and -7 by ELISA (Tab. 1). Because of the difference in growing of selected varieties in the region, 6 varieties from Tekirdağ centre, 5 varieties from Şarköy and 3 varieties from Uzunköprü were tested in the study. Survey studies revealed that 268 of 421 samples were infected at least by one serotype of grapevine leafroll associated viruses. The most widespread virus was GLRaV-1 (37.05 %) followed by GLRaV-3 (33.01 %), GLRaV-2 (7.83 %) and GLRaV-7 (4.03 %), respectively.

As a result, 191 of 421 tested samples were found infected by one serotype of the virus, however, 68 samples by 2 and 9 samples by 3 serotypes. The most widespread mixed infection type was by GLRaV-1 and GLRaV-3.

Grapevine leafroll associated virus infection distribution was as 134 out of 217 samples (61.75 %) in Tekirdağ centre, 97 of 117 samples (82.90 %) in Şarköy, Tekirdağ and 37 of 87 samples (42.52 %) in Uzunköprü district, Edirne, were found infected by tested viruses.

Considering the serotype infections, GLRaV-1 was found the most prevalent virus (43.31 %), followed by GLRaV-3 (23.96 %), GLRaV-2 (5.99 %) and GLRaV-7 (0.46 %) in Tekirdağ centre (Tab. 1).

As regarded for the varieties, infection ratios of grapevine leafroll associated viruses were the most higher in Alphonse lavalée (94.11 %), followed by Yapıncak (91.11 %), Hafızali (67.56 %), Cinsaut (48.27 %), Cardinal (43.47 %) and Semillon (37.83 %), respectively in Tekirdağ centre (Fig. 2). Yapıncak, a local variety, was found totally infected (100 %), followed with a lesser extent by Cinsaut (88.46%), Alphonse lavalée (83.3 %), Semillon (75.00 %) and Cardinal (63.63 %) in Şarköy (Fig. 2), Tekirdağ. Infections by GLRaVs was found highest in Alphonse lavalée (86.66 %) followed by Hafızali (43.58 %) and Cardinal (41.17 %) in Uzunköprü district, Edirne (Fig. 2).

GLRaV-1 was the most common virus serotype in Tekirdağ centre in Yapıncak (91.11 %) but very few infections were detected in Semillon (5.40 %). GLRaV-3 was commonly widespread in Alphonse lavalée (88.23 %), but the lowest infection ratio was found in Yapıncak variety (4.44 %). GLRaV-2 was found in Hafızali, but no infection detected in Cardinal and Cinsaut. GLRaV-7 was just detected in Yapıncak variety (2.22 %).

GLRaV-3 was found the most prevalent virus in Şarköy district, Tekirdağ (59.82 %), followed by GLRaV-1 (41.02 %), GLRaV-2 (12.82 %) and GLRaV-7 (11.11 %) (Tab. 1).

Table 1. Results of ELISA tests of grapevine leafroll associated viruses (GLRaV -1, -2, -3 and -7) in Thrace region in Turkey.

Variety	No of Tested Samples	Total Infected Samples	GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-7
			Tekirdağ Centre			
Alfonse Lavalée	17	16 (% 94.11)	11 (% 43.47)	1 (% 5.88)	15 (88.23)	0 (% 0)
Cardinal	23	10 (% 43.37)	8 (% 34.78)	0 (% 0)	8 (% 34.78)	0 (% 0)
Cinsaut	58	28 (% 48.27)	21 (%36.20)	0 (% 0)	14 (% 24.13)	0 (% 0)
Hafızali	37	25 (% 67.56)	11 (% 29.72)	10 (% 27.02)	4 (10.81)	0 (% 0)
Semillon	37	14 (% 37.83)	2 (% 5.40)	1 (% 2.70)	9 (24.32)	0 (% 0)
Yapıncak	45	41 (% 91.11)	41 (91.11)	1 (% 2.22)	2 (% 4.44)	1 (% 2.22)
Total	217	134 (% 61.75)	94 (% 43.31)	13 (% 5.99)	52 (% 23.96)	1 (% 0.46)
			Tekirdağ Şarköy			
Alfonse Lavalée	12	10 (% 83.33)	7 (% 58.33)	0 (% 0)	6 (% 50.0)	0 (% 0)
Cardinal	22	14 (% 63.63)	4 (% 18.18)	1 (% 4.54)	11 (% 50.0)	0 (% 0)
Cinsaut	26	23 (% 88.46)	13 (% 50.0)	8 (% 0)	22 (% 84.61)	0 (% 0)
Semillon	28	21 (% 75.0)	1 (% 3.57)	2 (% 0)	21 (% 75.0)	0 (% 0)
Yapıncak	29	29 (% 100.0)	23 (% 79.91)	4 (% 0)	10 (% 34.48)	13 (% 44.82)
Total	117	97 (% 82.90)	48 (% 41.02)	15 (% 12.82)	70 (%59.82)	13 (11.11)
			Edirne Uzunköprü			
Alfonse Lavalée	15	13 (% 86.66)	7 (% 46.66)	5 (% 33.33	3 (% 20.0)	0 (% 0)
Cardinal	17	7 (% 41.17)	3 (% 17.64)	0 (% 0)	6 (% 35.29)	0 (% 0)
Hafızali	39	17 (% 43.58)	4 (% 10.25)	0 (% 0)	8 (% 20.51)	3 (% 7.69)
Yapıncak	16	0 (% 0)	0 (% 0)	0 (% 0)	0 (% 0)	0 (% 0)
Total	87	37 (% 42.52)	14 (% 37.05)	5 (% 5.74)	17 (%19.54)	3 (% 3.44)
General Total	421	268 (% 63.65)	156 (% 37.05)	33 (% 7.83)	139 (% 33.01)	17 (% 4.03)

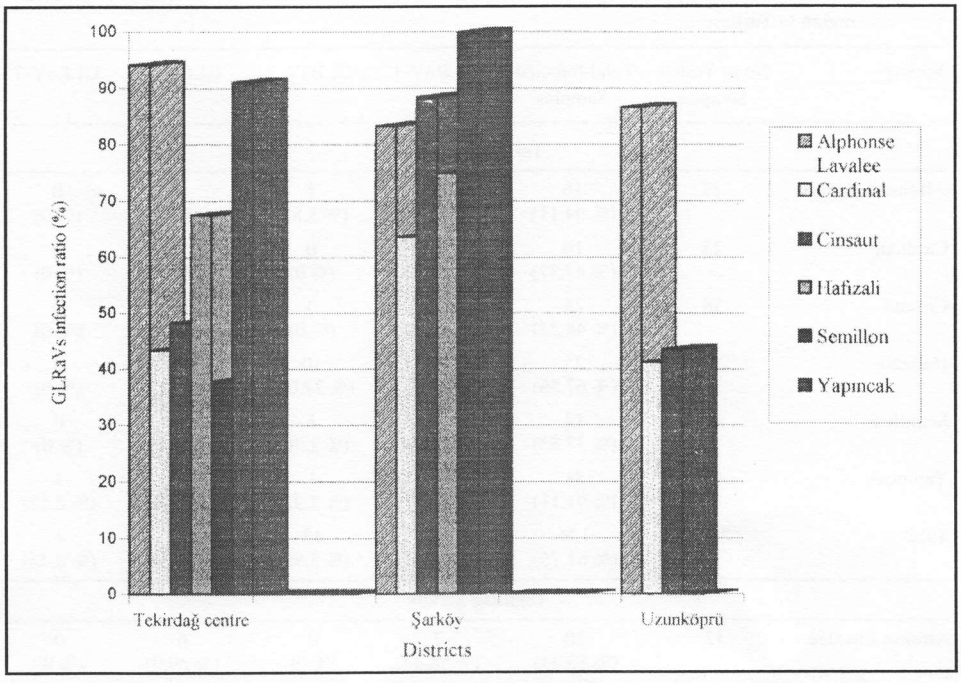

Figure 2. Incidence of grapevine leafroll associated viruses (GLRaVs) in some grape varieties grown Thrace region, Turkey.

GLRaV-1 was found the most prevalent virus (37.05 %), in Uzunköprü district, Edirne, followed by GLRaV-3 (19.54 %), GLRaV-2 (5.74 %) and GLRaV-7 (4.03 %) (Tab. 1).

GLRaV-3 was found at highest infection incidence (59.82 %) followed by GLRaV-1 (41.02 %), GLRaV-2 (12.82 %) and GLRaV-7 (4.82 %) in Şarköy district, Tekirdağ (Tab. 1).

As a result of the study, GLRaV-1 was prevalent in Yapıncak variety, GLRaV-3 in Cinsaut and Semillon varieties in Tekirdağ centre. However, surprizingly GLRaV-7 infection incidence was very high. GLRaV-2 has comparatively high infection ratio. Additionally Cinsaut and Semillon infected prevalently by GLRaV-1, Alphonse lavalée by GLRaV-3 in Şarköy district, Tekirdağ. High infection ratios were found for GLRaV-1 in Alphonse lavalée, GLRaV-3 in Cardinal and Hafızali varieties in Uzunköprü district, Edirne.

It is a need to carry out more detailed studies to determine the virus infections in the region. So that it may be possible to found virus free and high quality vines throughout the region.

ÖZET

TRAKYA BÖLGESİNDE YETİŞTİRİLEN BAZI ÜZÜM ÇEŞİTLERİNDE ASMA YAPRAK KIVRILMA HASTALIĞI İLE İLGİLİ VİRUSLARIN YAYGINLIĞININ SAPTANMASI

Trakya bölgesinde bazı üzüm çeşitlerinde asma yaprak kıvrılma virüs serotiplerinin yaygınlığının belirlenmesi amacıyla 1997 ve 1998 yıllarında bir survey çalışması yapılmıştır. ELISA testlerinde toplam olarak 421 örnek GLRaV-1, GLRaV-2, GLRaV-3 ve GLRaV-7 açısından değerlendirilmiştir. Toplam 421 bitkiden 268'i asma yaprak kıvrılması ile ilişkili virüs serotipleriyle infekteli bulunmuştur. En yaygın virüs serotipinin GLRaV-1 (% 37.05) olduğu, bunu sırasıyla GLRaV-3 (% 33.01), GLRaV-2 (% 7.83) ve GLRaV-7 (% 4.03)' nin izlediği belirlenmiştir. ELISA sonucunda 191 bitki en az 1, 68'i 2, 9'u 3 virüs serotipi ile infekteli durumda bulunmuştur. Bununla beraber 4 virüs serotipinin birlikte infeksiyonu belirlenmemiştir. GLRaV-1 ve GLRaV-3 birlikte infeksiyonları en sık rastlanan karışık infeksiyon durumu olarak bulunmuştur.

Anahtar kelimeler: Asma, Asma Yaprak Kıvrılma Virusu, Virus serotipleri, Trakya Bölgesi

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J. Turk. Phytopath., Vol. 29, No. 2-3, 95-103, 2000 ISSN 0378 - 8024

Studies on the Biological Control of Gray Mold Disease (*Botrytis cinerea* Pers.) of the Greenhouse Grown Tomatoes*

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ABSTRACT

The effectiveness of total of 185 microorganisms obtained from the tomato leaves in suppression of gray mold caused by *B. cinerea* was examined on broad bean and tomato plants. Ten days after inoculation with *B. cinerea* and antagonists, the incidence of infected plants was significantly reduced by five *Pseudomonas fluorescens* 77.56 (P.f. 144), 78.26 (P.f. 141), 80.51% (P.f. 163), 80.65 (P.f. 118) and 92.00 (P.f. 150)% respectively.

Key words: Gray mold, Biological control, fluorescent pseudomonads, tomato

INTRODUCTION

Botrytis cinerea is a destructive pathogen which causes severe losses in many crops. The pathogen initially invades senescent or dead plant tissue before it spreads to healthy plant tissue (Jarvis and Nuttall, 1981). Air temperature, relative humidity (RH) and duration of surface wetness effect the germination of spores and growth of the mycelium. High relative humidity in the greenhouse and free water on plant surface are the most significant environmental factors which influence infection by *B. cinerea*. The control of *B. cinerea* is based on the frequent application of chemicals because the control of the environmental conditions in many plastic greenhouse is not possible and cultivars of the greenhouse vegetables tomato, cucumber, pepper etc. resistant to gray mold are not available.

Gray mold is a serious problem in vegetable greenhouses and is often the reason for finishing a crop earlier than planned. The frequent application of fungicides such as benzimidazoles and dicarboximides which initially were highly effective against

* This study is a part of a project supported by TUBITAK.

B. cinerea have been available for years. The resistance of the pathogen to benzimidazole has developed in all areas of intensive use in many countries (Elad and Shtienberg, 1995).

The difficulty to manage gray mold has increased the need for alternative methods. *B. cinerea* must compete with other fungi and bacteria that are known to inhabit senescing petals and the crop phyllospere (Jarvis, 1997). Several of these microorganisms are antagonistic to *B. cinerea* and have been used in biological control experiments. Isolates of *Cladosporium cladosporiedes* (Eden et al., 1996), *C. herbarum* (Bhatt and Vaughan., 1963), *Gliocladium catenulatum* (Elad et al., 1993), *G. roseum* (Sutton et al., 1996), *Cryptococcus, Rhodotorulla, Xanthomonas, Bacillus, Lactobacillus, Pseudomonas* (Elad et al., 1993), *Bacillus firmus* and *Paecilomyces lilacinus* (Yıldız, 1990) have effectively suppressed gray mold.

The purpose of this study is to investigate the biological control of gray mold by certain bacteria and fungi to prevent the infection.

MATERIALS and METHODS

Material

Collection and isolation of the antagonistic microorganisms

The survey was carried out during the winter of 1998 in the tomato growing areas of Ege and Mediterranean region of Turkey. Uninfected leaves were randomly collected from each of 35 greenhouses of tomato plants. The samples were carried in the plastic bags to the laboratory.

Ten grams of intact leaves from each of samples were rinsed in 200 ml of distilled water and shaken at 90 rpm on a rotary shaker for a half hour at room temperature. The dilutions of the suspension were added to the each of three media (Martin, 1950, Kings B medium KB and NYDA) at nearly solidified condition. After 5 – 6 days, selected colonies under UV light for fluorescent pseudomonads were isolated and streaked on King B.The others were isolated on PDA and NYDA to obtain pure cultures. All 185 isolates were stored in refregirator until further use.

In – vitro Screening of the Cultures

In – vitro screening was carried out to assess the potential for fungal isolates to interact with *B. cinerea*. Dual cultures were set up by placing disks, 5 mm in diameter of the fungal candidate to be tested and *B. cinerea* at opposite sides of a 90 mm dish. The isolates that the mode of action is hyperparasitism were selected (Johnson et al., 1960).

Epiphytic yeasts were screened for their colony morphology and color at the NYDA medium.

In – vivo screening experiments

Faba bean plant cultivation (***Vicia faba* L.**): Faba bean plants were cultivated in the pots in the greenhouse conditions under natural day light. Plants were used in experiments when four full sets of leaves had developed (Delen et al, 1984, 1988; Jackson et al., 1997).

Tomato cultivation: Tomato (*Lycopersicum esculentum* RioGrande) plants were grown in the 20 cm pots in a greenhouse at 15 – 25°C under natural day light.

No fungicides were applied to both of crops.

Inoculum: *Botrytis cinerea* was grown on potato dextrose agar. Suspensions of conidia (1 X 10^5 conidia / ml) of *B. cinerea* were prepared from 10 – 14 days cultures by scraping the sporulating culture in sterile distilled water containing 1 % carrot juice and gelatin and filtering through three layered cheesecloth. Spore concentration was determined with a hemocytometre and adjusted as necessary.

Suspensions of fungal antagonists were prepared in the sterile distilled water. Cells scraped from the surface of 3 day old PDA cultures were suspended in sterile distilled water and concentrations were adjusted to 10^5 – 10^6 conidia per mililiter.

Yeast cells were prepared for 48 h on NYDB medium. The cultures were centrifuged at 4000 rpm for 10 minutes. The supernatant of each culture was poured down and the pellet was resuspended to a concentration of 10^9 cells / ml.

Bacterial inoculum was prepared in Kings – B medium. The cultures were shaken at 90 rpm for 48 h at approximately 25°C. Each culture was centrifuged as mentioned before. The bacterial isolate was resuspended to a concentration 10^9 and 10^{10} CFU/ml.

Treatment of Plant Material: The antagonistic yeast fungus and bacterial isolates were applied to whole plant by hand pulverizatior at a volume of 4 ml per plant. The biocontrol agents were applied two days before *B. cinerea*. The pots were covered with plastic bags to obtain higher relative humidity and incubated for 10 days (Delen et al., 1984, 1988).

Tomato tests for bacterial concentration: The most effective five bacterial isolates were selected and four concentrations of those isolates were sprayed to the tomato plants before *B. cinerea*. The bacterial isolates and their concentrations are presented at Figure 2.

Symptoms of the infected leaves were evaluated on a 0 – 5 scale (Anonymous, 1983) for broad bean plants and 0 – 4 scale for tomato plants (Anonymous, 1996). Experiments were arranged in randomized block design and repeated four times. All experiments were repeated at least twice.

RESULTS and DISCUSSION

***In–vitro* screening of fungal isolates**: Eight isolates of the fungus were evaluated by *in–vitro* tests. Antagonism between *B. cinerea* and only one isolate identified as *Gliocladium* sp. was defined as the hyperparasitic action against the pathogen.

Results of Broad bean

Yeast cultures: Thirty isolates of unidentifed white and pink isolates were screened on leaves of tomato plants. Screening was based on the effectiveness of the candidates on severity of symptoms of infected leaves. Some yeast cultures were found effective on broad bean plants (Table 1).

Bacterial isolates: One hundred eighty five fluorescent pseudomonads were isolated and selected under UV-light. Most of the bacterial isolates were found effective on broad bean and tomato plant tests (Table 2).

In the trials, four yeasts and twenty five bacterial isolates from the 185 isolates screened suppressed the development of *B. cinerea* on broad bean plant tests. Of these nine yeasts, four and of 29 fluorescent pseudomonads, inhibited the growth of *B. cinerea* over 60 % on broad bean plant tests. Those isolates were screened on the tomato plants.

When broad bean plants were inoculated with *Gliocladium* sp. and *B. cinerea* no disease suppression was occurred on the plants (30.00 %).

The effective isolates that were screened on the broad bean plants were tested with some fungicides. Imazalil and carbendazim + diethofencarb (50 µg / ml) were added to the medium to determine the susceptibility of fungi and bacteria. The yeast isolates that was found effective on the broad bean were influenced the fungicides of imazalil and carbendazim + diethofencarb and they were not selected for further tests.

Table 1. Effects of some yeast isolates on *B. cinerea* infection on broad bean plants

Isolates	Severity of the disease (%)	Effects of the isolates (%)	Isolates	Severity of the disease (%)	Effects of the isolates (%)
M 2 / 7	33.76	56.23	M 2 / 5	33.76	66.24
M 2 / 8	33.76	56.23	M 6 / 2	44.66	55.34
Control	77.14	-	M 6 / 3	44.66	55.34
			M 7	14.02	85.98
			M 11 / 1	16.20	83.79
			M 11 / 2	44.44	55.56
			M 19 / 2	31.03	68.97
			Control	100.00	

Table 2. Effects of some epiphytic fluorescent pseudomonads on *B. cinerea* infection on broad bean and tomato plants

Isolates	Broad beans		Tomato	
	Severity of the disease %	Effects of the isolates %	Severity of the disease %	Effects of the isolates %
FP 103	08.96	89.91	27.86 ab	-
FP 105	33.14	62.71	-	-
FP 106	12.56	85.86	16.72 ab	30.39
FP 107	04.16	95.31	17.65 ab	26.50
FP 109	24.80	72.09	-	-
FP 110	35.30	60.28	-	-
FP 117	29.58	66.71	-	-
FP 118	10.98	87.64	04.65 b	80.65
Control	88.88	-	-	-
FP 127	07.59	85.17	16.31 ab	32.09
FP 131	11.05	78.42	-	-
FP 132	10.30	79.88	08.01 ab	66.65
FP 133	15.61	69.51	-	-
FP 134	06.38	87.54	26.91 ab	-
FP 136	06.32	87.65	16.75 ab	30.26
FP 137	08.83	82.75	19.44 ab	19.06
FP 138	08.33	83.73	08.51 ab	64.57
FP 139	13.30	74.02	-	-
FP 140	13.37	73.89	-	-
FP 141	08.18	84.02	05.22 b	78.26
FP 142	04.91	90.41	20.83 ab	13.28
FP 143	08.58	83.24	33.12 ab	-
FP 144	07.37	85.60	05.96 b	77.56
FP 148	06.77	86.77	39.26 ab	-
FP 149	09.51	81.42	33.44 ab	-
FP 149	09.51	81.42	33.44 ab	-
FP 150	-	-	01.92 b	92.0
FP 162	10.99	78.53	24.96 ab	-
FP 163	09.22	81.99	04.68 b	80.51
FP 166	10.35	77.78	15.95 ab	33.59
Control	51.21	-	24.02 a	-

Significant differences $P < 0.05$. Values shown the means of two experiments

Tomato plant test results

The fluorescence pseudomonads were selected for the present activity on the basis of their effectiveness in reducing the disease rate on broad beans. Results of the most promising isolates are presented in Table 2. Bacterial isolates were found non-pathogenic to the plants as the hypersensitive reaction on tobacco plants.

The effective bacterial isolates were identified as *Pseudomonas fluorescens* by several analysis such as gram (-), levan colonies (-), oxidase (+), arginin dihydrolase (+), potassium hydroxide test (+), gelatin (+) and potato test (-) and some carbonhydrate tests such as Sucrose (+), Tartrate (-), Sorbitol (+) and Trehalose (+) (Fahy and Persley, 1983).

The bacterial isolates were also identified as *Pseudomonas fluorescens* by fatty acid analysis (Atatürk University, Faculty of Agriculture, Erzurum). The results of this Microbial Identification system are F.P. 118 (0.713 similarity index), F.P. 132 (0.775 similarity index), F.P. 138 (0.783 s.i.), F.P. 141 (0.868 s.i.), F.P. 144 (0.911 s.i.), F.P. 150 (0.837 s.i.), F.P. 163 (0.848 s.i.).

Under controlled conditions, *Pseudomonas fluorescens* 144, P.f. 141, P.f. 163, P.f. 118, P.f. 150 reduced the disease severity of tomato plants,inoculated with *B. cinerea* 77.56, 78.26, 80.51, 80.65 and 92.00 % respectively, compared to the check (Fig. 1).

Five bacterial isolates were tested with four concentration on the tomato plants. The most effective concentrations were established as 10^{10} CFU/ml, of FP 118, 144, 150, 163 and 10^{9} CFU/ml of FP 141 on tomato plants (Figure 2).

Five *P. fluorescens* were found effective on suppression of infection caused by *B. cinerea* on tomato plants.

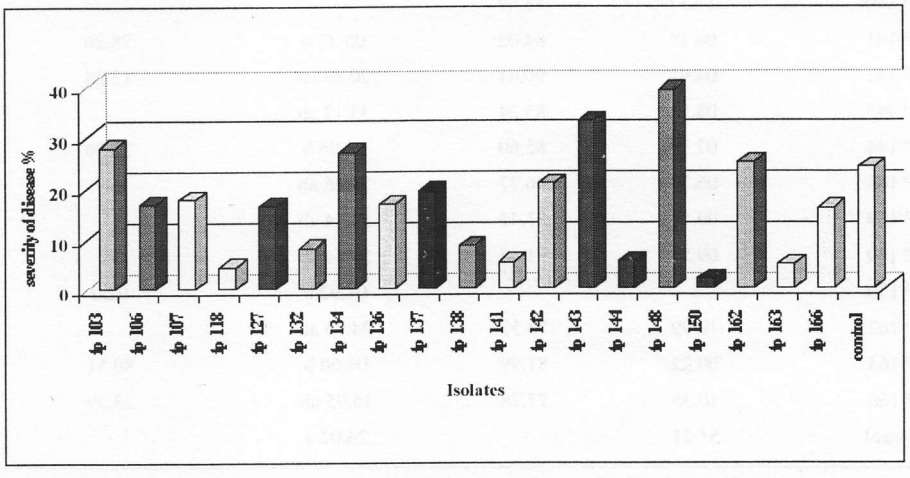

Figure 1. Effects of bacterial antagonists on *B. cinerea* infection on tomato plants

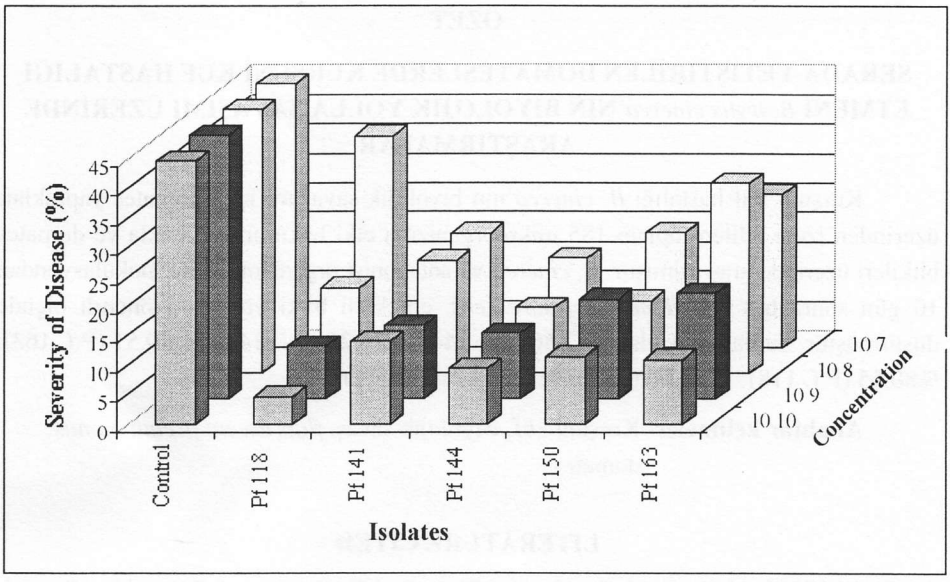

Figure 2. The effects of the dilution of *Pseudomonas fluorescens* on severity of the disease

In this present study, infection of tomato plants by *B. cinerea* was inhibited by some epiphytic microorganisms. A total of 185 fungal and bacterial isolates obtained from the surface of tomato leaves were evaluated for the ability to suppress *B. cinerea* disease on greenhouse grown tomatoes.

The infections caused by *B. cinerea* are severe problem in greenhouse vegetable crops (Jarvis, 1992; Malathrakis, 1989; Delen and Ozbek, 1992). Since cultural control methods fail to provide the effective control of the infection, growers continue to use fungicides to eliminate the pathogen (Elad et al, 1992, 1994 ; Yıldız and Delen 1985).

Sporulation of *B. cinerea* was reduced in the presence of the biocontrol agents. This was suggested earlier by Elad (1992, 1994); Gould et al (1996).

In the screening procedure twenty one bacteria were selected for their efficiency in protection the tomato plants against gray mold. The five bacteria were reduced the disease incidence in a range of from 77.56 % to 92.0 %. Promising results were obtained with five isolates of *Pseudomonas fluorescens* for gray mold disease caused by *B. cinerea*. A few studies have realized biological control on tomato by fluorescent pseudomonads (Gould et al, 1996).

In the present study, infection of tomato plants by *B. cinerea* was inhibited by some bacterial isolates with different degrees. Although the test results is promising the experiments should be proved under the greenhouse conditions.

ÖZET

SERADA YETİŞTİRİLEN DOMATESLERDE KURŞUNİ KÜF HASTALIĞI ETMENİ *Botrytis cinerea*'NIN BİYOLOJİK YOLLA SAVAŞIMI ÜZERİNDE ARAŞTIRMALAR

Kurşuni küf hastalığı *B. cinerea*'nın biyolojik savaşımı için, domates yaprakları üzerinden izole edilen toplam 185 mikroorganizma etki bakımından, bakla ve domates bitkileri üzerinde araştırılmıştır. *B. cinerea* ve antagonist organizmalar ile inokulasyondan 10 gün sonra beş *Pseudomonas fluorescens*, enfekteli bitki yüzdesini önemli ölçüde düşürmüştür, bunlar sırasıyla %77.56 (P.f. 144), %78.26 (P.f. 141), % 80.51 (P.f. 163), %80.65 (P.f. 118) %92.00 (P.f. 150) olarak bulunmuştur.

Anahtar kelimeler: Kurşuni küf, Biyolojik savaş, *fluorescent pseudomonas*, domates

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J. Turk. Phytopath., Vol. 29, No. 2-3, 105-111, 2000 ISSN 0378 - 8024

Characterization and numerical taxonomy of *Pseudomonas tolaasii* isolates using antimicrobial susceptibility patterns

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ABSTRACT

The susceptibilities of *Pseudomonas tolaasii* isolates (n= 160) isolated from sporophores of the *Agaricus bisporus* to 20 antimicrobial agents were determined by disk-diffusion method. *P. tolaasii* NCPPB 2192^T and NCPPB 2325 were used as reference strains. All *P. tolaasii* isolates were sensitive to tobramycine and kanamycine. Antimicrobial susceptibility pattern of isolates showed two distinct phenotypic group. Tetracycline sensitivity and glucose utilization were the most diagnostic characters between the phenotypic groups. The results showed that in addition to normal identification methods it is possible to differentiate some of the varieties of *P. tolaasii* with the help of antimicrobial susceptibility tests.

Key words: *Pseudomonas tolaasii*, antibiotics, numerical taxonomy

INTRODUCTION

Bacterial brown blotch (Tolaas 1915) caused by *Pseudomonas tolaasii* (Paine 1919) and a variant sometimes referred to as *P. gingeri* (Rainey *et al.*, 1992) is considered as the most common and serious bacterial disease on cultivated mushrooms throughout the world. Under some environmental conditions still not well-determined, but influenced by temperature and relative humidity, the bacterium can become pathogenic and provoke the brown blotch disease (Soler-Rivas *et al.*, 1999). Taxonomy of the *P. tolaasii* group is not fully resolved (Rainey *et al.*, 1992, Wells *et al.*, 1995). It is described in the Section V of Bergey's Manual of Systematic Bacteriology (Palleroni 1984) due to the natural relationships with well characterized species of the genus Pseudomonas are largely unknown.

The aim of the present study is numerical taxonomy of bacterial pathogens responsible for the brown discoloration on the *Agaricus bisporus* using antimicrobial susceptibility and biochemical tests data.

MATERIALS and METHODS

Isolation of bacteria

Sporophores of the *Agaricus bisporus* obtained from Manisa and İzmir province showing brown or reddish-brown blotches were used. Isolation of bacteria from altered caps or stipes was performing on King's medium B (KB) following the usual procedures (Lelliot and Stead, 1987). Pure cultures were screened for pathogenicity and for the "white line" reaction (Wong and Preece, 1979). For this purpose authentic strains of *P. tolaasii* and *P. "reactans"* NCPPB 1311^T were used.

Characterization & susceptibility testing

All the bacterial isolates were assayed for their ability to form a precipitate (white line reaction) in KB medium with *P."reactans"* NCPPB 1311^T. Auxanographic features were determined in API 20 E galleries (BioMerieux, Fr.).

Antimicrobial susceptibility of the isolates and reference strains was tested on plates of Mueller-Hinton agar (Difco) pre-inoculated with the test organism and dried (Bauer *et al.*, 1966; NCCLS, 1990). Antimicrobial susceptibility test discs (Oxoid) were placed on the agar surface. Following discs were used: Erythromycin 15 µg (E); Streptomycin 10 U (S); Polymyxin B 300 IU (PB); Penicillin G 10 IU (P); Kanamycin 30 µg (K); Tetracycline 30 µg (TE); Sulphamethoxazole-Trimethroprin 25 µg (SXT); Neomycin 30 µg (N); Chloramphenicol 30 µg (C); Doxycycline 30 µg (DO); Gentamycin 10 µg (CN); Carbenicillin 100 µg (CAR); Bacitracin 10 IU (B); Tobramycin 10 µg (TOB); Cefalexin 30 µg (CL); Ampillicin 10 µg (AMP); Rifampicin 5 µg (RD); Vancomycin 30 µg (VA); Nalidixic acid 30 µg (NA); Novobiocin 30 µg (NB).The plates were evaluated after 24 and 48 hours of incubation at 27 °C. If the area surrounding a disc (≥ 16 mm diameter) was free from bacterial growth, it was recorded that the organism was sensitive to that antibiotic. Tests were performed in triplicate. All susceptibility tests could be read without difficulty after 24h of incubation. Pure bacterial cultures were stored at -20 °C in glycerol. Reference strains of *P. tolaasii* (NCPPB 2192^T and NCPPB 2325) obtained from the National Collection of Plant Pathogenic Bacteria, UK and were used as controls.

Numerical taxonomy

Numerical analysis of the data obtained from antibiotic susceptibility and carbon utilization tests was performed by using the simple matching coefficient. The results of

tests that were positive or negative for all of the strains were eliminated from subsequent numerical analysis. The data matrix was used to estimate the strain similarities, with calculation of the simple matching coefficient, and cluster analysis was computed into phenogram by using complete linkage clustering method (Sneath and Sokal, 1973). Isolated strains were grouped under eighth clusters (close groups) and type strains. All analyses were carried out with the TAXON-X program (Chun, 1995).

RESULTS and DISCUSSION

Susceptibilities of isolated and two reference strains of *Pseudomonas tolaasii* (NCPPB 2192^T and NCPPB 2325) to antibiotics were determined. All strains were sensitive to tobramycine and kanamycine. Resistance for all investigated strains was noted for 11 antibiotics: rifampicin, cefalexin, ampicillin, carbenicillin, bacitracine, erythromycine, chloramphenicol, penicillin G, vancomycin, nalidixic acid, and novobiocin. Penicillinase production was detected all strains tested. The results of other differential antibiotic susceptibility patterns and some phenotypic properties were shown in Table 1. Similar results were reported by Richardson, 1993 and Vantomme *et al.*, 1987. Most of the reported strains of *P. tolaasii* is tetracycline sensitive, but more than 40 percent of isolated strains were resistant to this antibiotic. Our findings were supported this observation (Table 1). Although, antibiotic usage was not offical for mushroom cultivation in Türkiye the resistance pattern relatively high. This results may be strong evidence

Table 1. Differential characteristics of isolates based on their antimicrobial susceptibility pattern and some phenetic properties

	Type strains		Phenotypic Clusters		
	P. tolaasii NCPPB 2192	*P. tolaasii* NCPPB 2325	A4 -1	M1 & M2	Others
No. of strains	1	1	20	40	100
Gelatin hydrolysis	+	-	+	+	+
Glucose assimilation	-	+	+	-	-
Rhamnose assimilation	-	-	-	+	-
Resistance to antibiotics ($\mu g\ ml^{-1}$)					
Doxycycline (30)	S	S	S	50%	40%
Streptomycine (10)	S	S	R	50%	40%
Tetracycline (30)	S	S	S	R	40%
Polymyxine B (300)	R	R	S	-	40%
Gentamycine (10)	S	S	S	-	20%
SXT(*) (25)	R	R	S	R	R
Neomycine (30)	S	S	S	-	20%

S. Sensitive (≥ 16 mm diameter), **R**: Resistance, (*). Sulphamethoxazole-Trimethroprin
NCPPB: National Collection of Plant Pathogenic Bacteria, Hertsfordshire, U.K.

that probable transfer of resistance plasmids between Pseudomonas strains and other compost microflora.

Two main phenotypic cluster-groups, showed in Figure 1, were obtained after a computer-assisted numerical analysis from antibiotic susceptibility data. Group A; contains the type (NCPPB 2192 & NCPPB 2325) and tetracycline sensitive strains of *P. tolaasii* (B8-2, I-1, I-2, A4-1). Group B contains tetracycline resistant strains (M-1, M-2, I-3, I-4). Cluster-groups A and B except strains A4-1 and I-4 were defined at the 85% and 75% similarity (S) levels respectively. Strains A4-1 and I-4 do not share significant (S ≥75%) relationships with any of he cluster-groups (Fig. 1).

Glucose and gelatin is the most seperative carbon source for biochemical characterization. None of the strains utilized mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose and urea tested in API galleries.

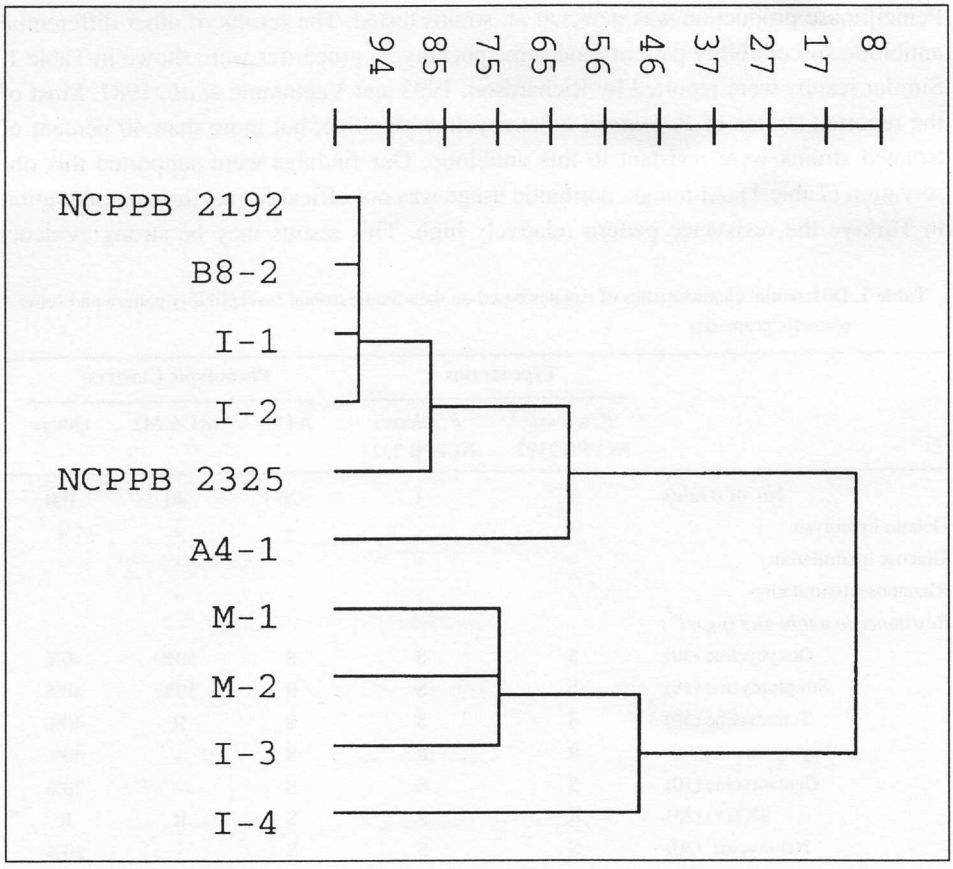

Figure 1. Phenogram showing the positions of *P. tolaasii* strains based on the antibiotic susceptibility tests. The scale shows the similarities in %.

Our study demonstrated clearly that antibiotic susceptibility patterns may be useful in distinguishing between similar strains of pathogenic *P. tolaasii*, occuring on cultivated mushrooms (Fig. 1). This method is more economical and will take a shorter time than classical tets. But, for a high reliable result, should be used with other taxonomical data.

The resistance or susceptibility to inhibitors are also generally stable characters and can serve as diagnostic aids. Furthermore, the patterns of susceptibility to antibiotics can be useful in distinguishing similar species from each other as well as the strains of the same species (Trüper and Schleifer, 1992). Studies done by Tamer and Bursalioglu, (1984); El-Banna, (1989); Şahin and Tamer, (1998) have shown that strains can be clearly differentiated based on the their antibiotic susceptibility pattern.

As a result, in addition to normal determination methods, it is possible to separate some of the varieties of *P. tolaasii* with the help of this technique. This technique may be useful for the detection of negative "white line reacting" strains of *P. tolaasii*.

ÖZET

Pseudomonas tolaasii İZOLATLARININ ANTİMİKROBİYAL DUYARLILIK PROFİLLERİ KULLANILARAK KARAKTERİZASYONU VE NUMERİK TAKSONOMİSİ

Agaricus bisporus sporoforlarından izole edilen (n= 160) *Pseudomonas tolaasii* suşlarının 20 antimikrobiyal maddeye karşı duyarlılıkları disk-diffüzyon metodu ile belirlendi. *P. tolaasii* NCPPB 2192^T ve NCPPB 2325 referans suşlar olarak kullanıldı. Tüm *P. tolaasii* izolatları tobramycin ve kanamycine duyarlıdır. İzolatların antibiyotik duyarlılık profilleri iki farklı fenotipik grubu gösterdi. Tetrasiklin duyarlılığı ve glukoz kullanımı fenotipik gruplar arasında en tanımlayıcı karakterler olarak belirlendi. Sonuçlar gösterdi ki, normal identifikasyon metodlarına ek olarak antimikrobiyal duyarlılık testleri yardımıyla *P. tolaasii*'nin bazı varyeteleri ayırd edilebilir.

Anahtar kelimeler: *Pseudomonas tolaasii*, antibiyotikler, numerik taksonomi

ACKNOWLEDGEMENTS

We are grateful to Dr. N. Sante Iacobellis (Universita degli studi della Basilicata, Italy) for helpful discussion and providing some *Pseudomonas tolaasii* strains used in this study. This work was partly supported by grants TBAG/AY-44 from TÜBITAK and AF077 from the Celal Bayar University.

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J. Turk. Phytopath., Vol. 29, No. 2-3, 113-119, 2000 ISSN 0378 - 8024

Studies on Spread and Characterization of the Citrus Chlorotic Dwarf Agent in the Eastern Mediterranean Region of Turkey

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ABSTRACT

Recently, a new virus-like disease of citrus transmitted by the Japanese bayberry whitefly, *Parabemisia myricae* (Kuwana) was discovered in citrus plantations of Turkey. This disease was named "Citrus Chlorotic Dwarf (CCD)" and is causing serious losses within a limited area. Natural spread was rapid at first, but recent surveys show that spread has slowed after the vector was put under biological control. A survey was conducted in newly established orchard in Icel area to determine the current infection rate of CCD. In total, 7,145 trees from 11 orchard were examined and average infection rate was found 7.8 % for all blocks. A procedure to partially purify the virus and recover infectivity from a specific region of sucrose gradients is described and attempts to recover greater yields are in progress. Circumstantial evidence suggests that the virus is stable, and phloem limited. It is present only in low concentrations in infected citrus plants or is very difficult to extract from infected tissues.

Key words: Citrus chlorotic dwarf, purification, survey, whitefly

INTRODUCTION

A new citrus virus-like disease, named citrus chlorotic dwarf (CCD), was first discovered in Turkey in the late 1980s, in 20 year old Minneola tangelo and Kutdiken lemon trees (Cinar et al., 1993). Within a short period large numbers of trees became infected within a limited area in the East Mediterranean region of Turkey where about 70 % of the total citrus production is concentrated. The disease is transmitted from citrus to citrus by Japanese bayberry whitefly, *Parabemisia myricae* (Kuwana) (Cinar et al., 1994; Korkmaz et al., 1994), which was introduced into Turkey several years prior to the first appearance of CCD. After the bayberry whitefly populations were reduced to low levels by biocontrol agents (Sengonca et al., 1998), the incidence of new CCD infections has seemed to be slow.

The causal agent is graft-transmissible and can be transmitted experimentally by stem-slash inoculation between citrus plants (Korkmaz et al., 1995). It has not been

mechanically transmitted by sap inoculation of the leaves between citrus hosts or to herbaceous plants. Electron microscopy of tissue extracts has failed to reveal distinctive virus-like particles and no dsRNA has been found in plants infected only with CCD. No virus-like inclusions suggestive of a gemini virus infection have been found in the infected plant cells (Korkmaz, 1997). While the causal agent remains poorly characterizated, circumstantial evidence suggests that it is probably phloem-limited virus that may be present in low concentrations.

This paper reports surveys for new infections of CCD in recently planted virus-free orchards in the CCD affected region of Turkey. New information on the purification and characterization of the causal agent is also presented.

MATERIALS and METHODS

Survey work: A survey was done in newly established orchard in the Icel area where CCD is common in older trees to determine the current rate of infection of CCD. Orchards were randomly selected from a group of plantings that were at least two years old and had been established with known sources of virus-free trees. Each tree in selected orchards was surveyed for CCD symptoms. Survey work was not done in Adana and Hatay provinces due to absent or very low infection of CCD in that region. Totally, 7,415 trees from 11 orchards were examined. The nearest adjacent old orchards established by traditional methods were also checked for CCD symptoms to determine the nearest CCD-infected trees. Some citrus nurseries were also surveyed for CCD and other problems in Icel province.

Purification: Purification studies were conducted with tender new flush tissue harvested from plants systemically infected by graft or stem-slash inoculation with an isolate of CCD obtained from a citrus seedling infected experimentally by the bayberry whitefly. Unless noted otherwise, bark, tender shoot tips and young symptomatic leaves were pooled for extraction. For most tests, tissue was extracted in three vol. of cold 0.05 M potassium phosphate buffer containing 10 mM DTT (di-thiothreitol) and adjusted to pH 7.4. Tissue was homogenized in a Waring Blendor for approximately 1 min and then for an additional minute at 20,000 RPM with a Polytron dispersion homogenizer equipped with a 45 mm generator. The homogenate was filtered through cheesecloth and centrifuged for 15 min at approximately 15,000 g. The supernatant was collected and clarified by mixing with a 1:1 mixture of cold n-butanol and chloroform or mixed with polyethylene glycol 6,000 (PEG) and NaCl to final concentration of 6% (weight/vol) and 0.15 M respectively. Incubations periods were approximately 30 min for butanol-chloroform and 2-12 hours for PEG. Extracts mixed with butanol-chloroform were centrifuged for 10-15 minutes at 10,000 RPM and the aqueous phase was recovered for further treatment. PEG pellets were resuspended in 1/10 of the original vol. of extraction

buffer for at least two hours by stirring and centrifuged to pellet debris. In some cases a second cycle of PEG precipitation was used. The calcium phosphate gel method for purification of Citrus Variegation Virus (CVV) (Garnsey, 1974) was also used in limited tests.

Several types of sucrose gradients were used for rate zonal centrifugation, including step (Matthews, 1991), truncated linear and full length linear gradients. Gradients were layered by hand or by a gradient forming device. Gradients were centrifuged in a Beckman Sw28 rotor at 4°C and fractionated manually with a syringe device or with an Isco Density gradient fractionator. Partially purified preparations of CVV (Garnsey, 1974) and Tobacco Mosaic Virus (TMV) were used as markers in rate zonal centrifugation studies.

Infectivity assays were done by slash cut inoculation of Alemow (*Citrus macrophylla*) seedlings of 50-60 cm tall. Approximately 100 cuts were made with a scapel blade moistened with the inocula. Thirty to 40 µl of inoculum was used for each plant and normally six plants were inoculated per treatment. Plants were cut back periodically to force new growth and examined for symptom developments.

RESULTS and DISCUSSION

Spread of CCD in new plantings. The results of surveys conducted for CCD in recently planted orchards is summarized in Table 1. CCD-infected trees were found in 8 of 11 orchards. The highest infection rate (56 %) was found in a 6 year-old Satsuma orchard in Tarsus (Yunusoglu village). The satsuma trees were planted among 25 year-old Kutdiken lemon trees heavily infected with CCD. The lemon trees were subsequently removed, but CCD was apparently transmitted quickly by *P. myricae* from lemon trees to the nearby Satsuma trees. No infected trees were found in one 3 year-old Satsuma orchard and 4 year-old Washington Navel sweet orange and Marisol mandarin orchards (Table 1, Orchards No: 7, 8 and 10). The non-infected Satsuma orchard was established far away from a source of CCD infection. This orchard had citrus plants on one side that were not infected with CCD. The Marisol and Washington Navel varieties are tolerant to CCD and symptoms may not have been present, even if trees had been infected. Only one infected tree with CCD symptoms was found in a 4 year-old Satsuma orchard, and 3 year-old Kutdiken and Interdonato lemon orchards (Table 1, Orchards no:1,5,9). Those orchards were established 100 m from the nearest infected citrus orchards which did not have high infection rates. The average infection rate for all blocks was 7.8 %. This was quite a high infection rate for 3-6 year-old orchards, given the low populations of *P. myricae* present. While *P. myricae* is the only vector identified, further tests with other whiteflies present in the area such as citrus whitefly, *Dialeurodes citri* (Ashmed) and the cotton whitefly, *Bemisia tabaci* (Genn) are warranted.

Table 1. Infection rate of citrus chlorotic dwarf in new established orchards near Icel, Turkey

No	Place	Varieties	Age	IT/Total*	Infection rate (%)
1	Yenice-TARSUS	Satsuma	4	1/650	0.15
2	Yenice-TARSUS	Ruby-Red	5	5/300	1.66
3	Karacailyas-IÇEL	Satsuma	5	66/446	14.79
4	Ozbek-TARSUS	Satsuma	3	42/738	5.69
5	Resadiye-TARSUS	Kütdiken	3	1/755	0.11
6	Yunusoglu-TARSUS	Satsuma	6	318/566	56.18
7	Cagbasi-TARSUS	Satsuma	3	0/704	0
8	Yenice-TARSUS	W.Navel	4	0/850	0
9	Aliaga-TARSUS	Interdonato	4	1/618	0.16
10	Resadiye-TARSUS	Marisol	4	0/715	0
11	Ozbek-TARSUS	Star-Ruby	4	149/1073	13.88
			Total	583/7415	7.86

All trees in survey areas established from sources free of CCD infection
*Infected trees/All surveyed trees

Table 2. Infectivity tests for CCD following extraction, precipitation with PEG 6000 and density gradient centrifugation.

Inoculum	No. plants inoculated	No. plants infected
Extract*	6	1
1st PEG treatment**	6	2
2nd PEG treatment***	6	4
Density gradient Fraction 7****	6	3

* Extract tested following low speed centrifugation
** Inoculum was PEG pellet resuspended in 1/10 original vol. of buffer and clarified by low speed centrifugation
*** Assay after 2nd cycle of PEG precipitation
**** Assay of fraction recovered from sucrose density gradient in region between CVV and TMV zones in comparable tubes.
All assays by stem slash inoculation (100 cuts) to seedlings of *C. macrophylla.*

Nursery studies

Eleven citrus nurseries were surveyed for CCD in Icel province and all were found infected with CCD. These are small family owned nurseries having between 3000-30000 seedling capacity. Citrus plant production is made by traditional methods from local sources of budwood which have several viruses and viroids present in

addition to CCD. Some nurserymen know that CCD is a virus disease, but no one has removed infected trees from their nurseries or stopped selling infected trees to the growers.

Purification studies

Extracts obtained by vigorous homogenization of tissue were more infectious than extracts prepared by gentler methods suggesting that the causal agent was not an easily fragmented rod-shaped particle. Infectivity was detected in resuspended PEG pellets and the infectivity was greater than in the initial extracts, suggesting substantial recovery of the "virus" in the original extract. Infectivity was also detected in extracts that were clarified with butanol-chloroform and then concentrated by high speed centrifugation or PEG precipitation. Infectivity was also detected in extracts prepared from frozen tissue, but infectivity was found lower.

Virus-specific zones were not seen rate zonal gradients loaded with preparations from CCD infected tissue, however, infectivity was detected from a region between the zone areas for CVV and TMV centrifuged in comparable gradients. Electron microscopy of zones with infectivity contained several types and sizes of isometric particles. We did not detect infectivity in preparations purified using the calcium phosphate gel clarification method previously used successfully for CVV.

Observations that the infectious agent is not removed from suspension by centrifugation at 20,000 x g for 15; remains infectious after treatment with butanol-chloroform; is precipitated in the presence of 6% PEG 6000, and sediments in rate zonal gradients similarly to other plant viruses supports the hypothesis that the casual agent of CCD is virus. Failure to achieve significant yields of virus suggests that the virus is present in low concentrations or is difficult to extract from infected tissues. It is also possible that the procedures used resulted in extensive loss of virions in the purification process. Further studies are in progress to improve the purification process and obtain sufficient quantities of virus for characterization and development of rapid detection probes.

Unless steps are taken to improve nursery practices, the citrus industry in Turkey will have to be increasingly serious virus disease problems. There are currently no official budwood certification programs or regulations in Turkey. However, a program to develop sources of virus-free sources of major cultivars and produce commercial quantities of high quality nursery trees has been established at the Subtropical Fruits Research and Experimental Centre of Cukurova University. In addition to currently supplying approximately 100,000 virus-free trees each year the program is a resource for clean budwood and modern nursery technology that could be used more widely.

STUDIES ON SPREAD AND CHARACTERIZATION OF THE CITRUS CHLOROTIC DWARF AGENT IN THE EASTERN MEDITERRANEAN REGION OF TURKEY

ÖZET

DOĞU AKDENİZ BÖLGESİ'NDE (TÜRKİYE) TURUNÇGİL KLOROTİK CÜCELEŞME HASTALIĞININ YAYILIŞI VE TANILANMASI ÜZERİNE ARAŞTIRMALAR

Ülkemizin turunçgil alanlarında son yıllarda Japon Defne Beyazsineği *Parabemisia myricae* ile taşınan virüs-benzeri bir hastalık etmeni tanımlanmıştır. Belirli bir bölgede önemli kayıplara neden olan hastalık "Turunçgil Klorotik Cüceleşme" olarak adlandırılmıştır. Hastalığın yayılması başlangıçta çok hızlı olurken daha sonra özellikle vektör böceğin biyolojik kontrolle baskı altına alınmasıyla yavaşlamıştır. Hastalığın yoğun olduğu İçel ilinde yeni kurulan bahçelerde bir sörvey çalışması yürütülmüştür. Toplam 11 bahçede 7.415 ağaç tek tek incelenmiş ve ortalama infeksiyon oranı % 7.8 olarak bulunmuştur. Kısmen purifiye edilen preparasyondan sukroz density gradient sonucunda spesifik bir bölgeden infektiöz partiküller elde edilmiş ve çalışmalar elde edilen bu partiküllerin konsantrasyonunun artırılması yönünde olmuştur. Yapılan çalışmalar sonucu elde edilen bulgular virüsün stabil ve floemde sınırlı olduğunu göstermektedir. Etmen infekteli turunçgil bitkisinde çok düşük konsantrasyonda bulunmakta veya infekteli dokulardan ekstraksiyon çok zor olmaktadır.

Anahtar kelimeler: Turunçgil klorotik cüceleşme, pürifikasyon, sörvey, beyazsinek

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J. Turk. Phytopath., Vol. 29, No. 2-3, 121-131, 2000 ISSN 0378 - 8024

Comparison of the Formats of Three ELISA (PAS-ELISA, ACP-ELISA, Indirect ELISA Kit) and Reagents for Detection of Some Viruses Infecting Cucurbits

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ABSTRACT

Formats of protein- A ELISA (PAS-ELISA), antigen coated plate ELISA (ACP-ELISA), and indirect ELISA kit were evaluated and compared for their usefulness in detection of cucumber mosaic virus (CMV), papaya ringspot virus type -W (PRSV-W), squash mosaic virus (SqMV), watermelon mosaic virus (WMV), and zucchini yellow mosaic virus (ZYMV). CMV was detected by CMV antiserum without any cross-reaction in three ELISA formats. SqMV was detectable by this antiserum in PAS-ELISA, not in ACP-ELISA and indirect ELISA kit formats. The three potyviruses, PRSV-W, WMV, and ZYMV, reacted with antisera of these viruses and gave cross-reaction with all three antisera in three ELISA formats. Our results indicated that indirect ELISA kit was more suitable to detect cucurbit viruses (CMV, PRSV-W, WMV, ZYMV) in field surveys and sample diagnosis because of its speed and usefulness; however, PAS-ELISA was more convenient to detect SqMV.

Key words: PAS-ELISA, ACP-ELISA, Indirect ELISA, CMV, ZYMV, WMV, PRSV-W, SqMV, ELISA formats

INTRODUCTION

Cucumber mosaic virus, the watermelon strain of papaya ringspot virus, squash mosaic virus, watermelon mosaic virus and zucchini yellow mosaic virus cause diseases of major importance in cucurbit in Turkey and worldwide (Campell, 1971; Lisa and Lecoq, 1984; Chala et al, 1987; Provvidenti et al., 1989; Sammons et al., 1989; Perring et al, 1992; Wang et al., 1992; Gera, 1994; Robinson et al, 1993; Yılmaz, 1999).

CMV is the type member of cucumovirus group which is transmitted by many aphid species in non-persistent manner (Francki et al., 1970). PRSW-W, WMV and ZYMV are flexuous rod-shaped potyviruses and produce pinwheel and scroll inclusion bodies in cytoplasm of their host (Lisa and Lecoq, 1984; Provvidenti, 1996a). These

potyviruses are transmitted by aphids in non-persistent manner (Purcifull et al., 1984; Blau and Perring, 1989; Ferreres et al., 1992; Provvidenti, 1996b; Wang et al., 1992). SqMV, a member of comovirus group, is a multicomponent, beetle transmitted, seed borne virus having a narrow host range (Hiebert and Purcifull, 1981; Nolan and Campbell, 1984).

Since the introduction of enzyme linked immunosorbent assay (ELISA) for detection of plant viruses (Clark and Adams, 1977), several ELISA procedures have been developed to decrease non-specific reaction and increase sensitivity and speed in the assays. These modifications include the ELISA systems employing F (ab`) fragments (Barbara and Clark, 1982), using antibodies from different animals (Van Regenmortel and Burckard, 1980), coating plates direct antigens (Mowat, 1971), using an initial protein A for coating plates (Edwards and Cooper, 1985), amplifying the enzyme substrate (Torrance, 1987; Van den Heuvel and Peter, 1989), using different substrates (Dietzgen et al., 1987), replacing chromogenic by fluorogenic substrates (Torrance and Jones, 1982), using semi-quantitative biotin streptavidin (Dietzgen and Herrington, 1991).

ELISA is used for many different purposes in plant biology. Presence of a sensitive ELISA system can facilitate to screen large number of plant samples for diagnosis and give information about the nature of infection (Campbell, 1971; Nolan and Campbell, 1984). ELISA is very useful technique especially in quantifying viral antigens (Hobbs et al., 1987; Koch and Salomon, 1994) and differentiating resistant and tolerant lines in plant breeding programs (Dietzgen and Herrington, 1991). The purpose of this study is to determine the best ELISA format for detection of cucurbit viruses by comparing Protein A ELISA (PAS-ELISA), Antigen coated plate ELISA (ACP-ELISA), and commercial ELISA kit (from Agdia).

MATERIALS and METHODS

Viruses

The isolates of CMV, PRSV-W, and WMV were supplied by J. L. Sherwood, University of Georgia. SqMV and ZYMV were kindly provided by Dr. R Gergerich, University of Arkansas. CMV was maintained in *Nicotina tabacum cv. Xanthi*-nc (Francki et al., 1970) and each of the other viruses was maintained in *Cucurbita pepo* (Campbell, 1971; Purcifull et al., 1984).

Antisera

The polyclonal antibodies to CMV isolates (CMV-9, CMV-C) and SqMV were obtained from Dr. R Gergerich, University of Arkansas. Antibodies to other isolates (PRSV-W, WMV and ZYMV) had been previously prepared in the laboratory of J. L.

Sherwood. These antibodies were used in PAS-ELISA and ACP-ELISA. Antibodies and antibody conjugates to CMV, PRSV-W, SqMV, WMV, and ZYMV were also bought from Agdia (Elkhart, Indiana, USA) for use indirect ELISA according to the supplier.

Reagents

The following buffers were used: Phosphate buffered saline (0.002 M phosphate, 0.15 M NaCl, at pH 7.4) containing 0.5 ml / L Tween -20 (PBST); coating buffer containing 0.05 M sodium carbonite at pH 9.6; extraction buffer [0.015 M sodium carbonate, 0.03 sodium bicarbonate, and 20 gr. / L polyvinylpyrrolidone (PVP) MW 40000 at pH 9.8]; ECI buffer containing PBST plus 2 gr. / L bovine serum albumin (BSA), 20 gr. / L PVP at pH 7.4; Substrate buffer (10 % diethonolamine adjusted to pH 9.8 using HCl). All buffers contained 0.02 % sodium azide as preservatives (Hiebert and Purcifull, 1981).

ELISA sample preparation

Samples (0.1 gr/ml) were ground in a mortar with 0.02 M PBS-T at pH 7.4 for PAS-ELISA, with carbonate buffer at pH 9.6 for ACP-ELISA and with ECI buffer at pH 9.8 for indirect ELISA kit.

ELISA Procedures

Protein-A sandwich ELISA (PAS-ELISA)

Protein-A was diluted in coating buffer (pH 9.6) at a concentration of 1 µg / ml and added to plates as described by Edwards and Cooper (1985). An optimum concentration of antisera in PBST buffer (1/ 1000) was added to wells. Then, plant samples were added to wells at 1/10 and 1/100 dilutions. The secondary antibody was added to wells the same as the primary antisera concentration. After that, conjugated protein-A was added at 1/1000 dilution. This was followed by adding p-nitrophenyl phosphate in diethanolamine substrate buffer. In each step, plates were washed 3X with PBST. The absorbance values was read at 405 nm by a microplate reader in 30 and 45 minutes.

Antigen coated plate ELISA (ACP-ELISA)

Diseased and healthy plant samples were ground in mortar with coating buffer, at pH 9.6 (Mowat, 1984). A 1/1000 dilution of antiserum was added to wells. Then, wells were exposed to anti-rabbit alkaline phosphotase at 1/32000 dilution. After that, p-nitrophenyl phosphate in dietholamine substrate buffer was added. The absorbance values were read at 405 nm, by a microplate reader in 30 and 45 minutes.

Indirect ELISA kit (from Agdia)

The indirect ELISA kit assays were performed according to manufacturer instructions, which were essentially similar to ACP-ELISA format with the following

modifications. Samples were ground in extraction buffer at pH 9.8, loaded into wells and incubated for 1 hr. Plates were washed with the PBST between all incubation steps. A 1/ 200 dilution detection antibody was added and incubated for 2 hrs. This was followed by adding alkaline phosphotase conjugated antibody at the same dilution. Finally; p-nitrophenyl phosphate in diethanolamine substrate buffer was loaded into wells and absorbance values was read at 405 nm, by a microplate reader in 30 and 45 minutes (Agdia kit procedure, Elkhart, Indiana, 1996).

RESULTS

PAS-ELISA

An optimum concentration of protein-A (1 µg / ml) was used to detect CMV, PRSV-W, SqMV, WMV, and ZYMV in PAS-ELISA system as described by Edwards and Cooper (1985). The virus specificity of PAS-ELISA format using antisera to CMV, PRSV-W, SqMV, and ZYMV, respectively, was detected from infected plant sap as well as healthy plant sap as control (Table 1). CMV was detectable by CMV-9 antiserum at 1/ 10, 1/ 100 dilutions in PBST without any cross-reaction to other cucurbit viruses used

Table 1. Detection of CMV, PRSV-W, SqMV, WMV, and ZYMV in green house-grown test plant sap using Protein- A ELISA

			Antibodies*					
Virus**	Plant	Dilutions	CMV-9	CMV-C	PRSV	SMV	WMV	ZYMV
	Infected	1/10	**0.480**	**2.000**	0.074	0.007	0.118	0.273
CMV		1/100	**0.489**	**2.000**	0.055	0.019	0.089	0.239
	Healthy	1/10	0.006	0.259	0.088	0.014	0.053	0.050
		1/100	0.001	0.197	0.078	0.015	0.055	0.002
	Infected	1/10	0.000	0.087	**1.710**	0.037	**2.000**	**0.554**
PRSV		1/100	0.000	0.069	**1.402**	0.040	**1.326**	**0.303**
	Healthy	1/10	0.001	0.085	0.195	0.037	0.026	0.069
		1/100	0.006	0.069	0.058	0.001	0.006	0.001
	Infected	1/10	0.021	0.815	0.051	**2.000**	0.040	0.096
SqMV		1/100	0.002	0.312	0.033	**2.000**	0.046	0.083
	Healthy	1/10	0.004	0.783	0.069	0.021	0.011	0.003
		1/100	0.003	0.486	0.056	0.005	0.008	0.022
	Infected	1/10	0.004	0.640	**1.081**	0.108	**1.611**	**0.402**
WMV		1/100	0.007	0.447	**1.086**	0.070	**0.580**	**0.248**
	Healthy	1/10	0.006	0.518	0.066	0.020	0.029	0.003
		1/100	0.002	0.313	0.011	0.001	0.002	0.022
	Infected	1/10	0.016	0.525	0.009	0.010	0.190	**1.846**
ZYMV		1/100	0.006	0.572	0.015	0.003	0.098	**1.911**
	Healthy	1/10	0.007	0.438	0.000	0.015	0.009	0.023
		1/100	0.022	0.167	0.006	0.001	0.004	0.025

* Each of the antisera used in this assay was diluted 1/ 1000 concentration

** Absorbance values of specimens were measured at 405 nm after 45 min. substrate incubation and three times of the values of the negative controls have been considered as positive.

in this assay. CMV-C antiserum gave higher absorbance values in detection of CMV, but it cross-reacted both healthy plant tissues and other cucurbit viruses. The PAS-ELISA employing SqMV antiserum was very specific to detect SqMV and no reaction was detected with plant tissues and other cucurbit viruses. PRSV-W antiserum gave strong reaction with this virus and slightly strong reaction with WMV. Papaya ring spot virus and watermelon mosaic viruses were strongly detected by WMV antiserum. ZYMV antiserum gave very high absorbance to this virus, but the same antiserum showed a slight cross-reaction with WMV, PRSV-W, and CMV.

ACP-ELISA

Specificity of detection of CMV, PRSV-W, SqMV, WMV, and ZYMV using ACP-ELISA ELISA system was determined (Table 2). From the previous experiment, antibody concentration (1 µg / ml) was determined and used in this assay.

CMV-9 polyclonal antiserum reacted with only cucumber mosaic virus; however, CMV-C reacted with all cucurbit viruses as well as healthy plant tissues. Papaya ringspot virus type-W and watermelon mosaic virus were strongly detected by all three

Table 2. Detection of CMV, PRSV-W, SqMV, WMV, and ZYMV in green house-grown test plant sap using antigen coated plate ELISA

			Antibodies*					
Virus**	Plant	Dilutions	CMV-9	CMV-C	PRSV	SMV	WMV	ZYMV
CMV	Infected	1/10	**0.302**	0.882	0.425	0.053	**0.443**	**0.423**
		1/100	**0.266**	0.785	0.295	0.009	**0.382**	**0.275**
	Healthy	1/10	0.029	0.482	0.176	0.000	0.016	0.024
		1/100	0.006	0.477	0.152	0.003	0.001	0.018
PRSV	Infected	1/10	0.050	0.816	**1.284**	0.012	**1.356**	**1.289**
		1/100	0.055	0.780	**1.409**	0.005	**1.292**	**1.055**
	Healthy	1/10	0.037	1.075	0.334	0.001	0.040	0.030
		1/100	0.048	0.754	0.345	0.005	0.012	0.068
SqMV	Infected	1/10	0.046	0.792	0.429	0.027	0.036	0.002
		1/100	0.031	0.560	0.243	0.080	0.029	0.006
	Healthy	1/10	0.013	0.783	0.261	0.018	0.037	0.006
		1/100	0.001	0.769	0.255	0.006	0.021	0.013
WMV	Infected	1/10	0.070	0.799	**1.101**	0.024	**0.827**	**0.811**
		1/100	0.065	0.536	**1.278**	0.040	**0.926**	**0.965**
	Healthy	1/10	0.016	0.745	0.245	0.006	0.000	0.011
		1/100	0.037	0.879	0.234	0.009	0.001	0.024
ZYMV	Infected	1/10	0.050	1.023	0.270	0.023	**1.119**	**1.458**
		1/100	0.073	1.117	0.286	0.011	**1.114**	**1.429**
	Healthy	1/10	0.002	0.876	0.340	0.023	0.013	0.033
		1/100	0.001	0.719	0.100	0.005	0.015	0.001

* Each of the antisera used in this assay were diluted at 1/1000 in PBST

** Absorbance values of specimens were measured at 405 nm after 45 min. substrate incubation and three times of values of the negative controls have been considered as positive.

potyvirus antisera (PRSV-W, WMV, ZYMV). Zucchini yellow mosaic virus strongly reacted with ZYMV and WMV antisera, but slightly reacted with PRSV antiserum. CMV was slightly detected by all three potyvirus antisera. SqMV antiserum did not reacted with any cucurbit viruses and plant tissues and did not work in this assay.

Indirect ELISA kit

Detection specificity of indirect ELISA kit for cucurbit viruses was determined (Table 3). Cucumber mosaic virus specifically reacted with CMV antiserum without any cross-reaction to other cucurbit viruses used in this assay. SqMV did not reacted with neither viruses nor plant tissues. WMV antiserum gave higher absorbance values in detection of WMV; however, the same antiserum cross-reacted with PRSV-W and ZYMV. Papaya ringspot virus type-W was determined by both PRSV-W and WMV antisera. ZYMV antiserum specifically reacted with zucchini yellow mosaic virus.

Table 3. Detection of CMV, PRSV-W, SqMV, WMV, and ZYMV in green house -grown test plant sap using indirect ELISA kit

			Antibodies*				
Virus**	Plant	Dilution***	CMV	PRSV-W	SMV	WMV	ZYMV
CMV	Infected	1/10	**0.148**	-	-	-	-
		1/100	**0.281**	0.042	0.001	0.098	0.009
	Healthy	1/10	0.021	0.001	0.01	0.018	0.001
		1/100	0.001	0.001	0.001	0.001	0.001
PRSV	Infected	1/10	-	**1.131**	-	-	-
		1/100	0.016	**0.989**	0.017	**1.837**	-0.008
	Healthy	1/10	0.001	0.001	0.001	0.001	0.001
		1/100	0.011	0.011	0.009	0.001	0.001
SqMV	Infected	1/10	-	-	0.022	-	-
		1/100	0.001	0.02	0.016	0.056	0.004
	Healthy	1/10	0.001	0.001	0.002	0.001	0.001
		1/100	0.002	0.002	0.002	0.002	0.002
WMV	Infected	1/10	-	-	-	1.998	-
		1/100	0.001	**0.657**	0.001	**1.799**	0.022
	Healthy	1/10	0.001	0.001	0.001	0.001	0.001
		1/100	0.001	0.001	0.001	0.001	0.001
ZYMV	Infected	1/10	-	-	-	-	**0.256**
		1/100	0.011	0.011	-0.006	**1.151**	**0.216**
	Healthy	1/10	0.001	0.001	0.001	0.100	0.018
		1/100	0.001	0.001	0.001	0.046	0.011

* Each of the antisera used in this assay were diluted at 1/200 in ECI buffer.

** Absorbance values of specimens were measured at 405 nm after 45 min. substrate incubation and three times of values of the negative controls have been considered as positive.

*** To save antisera only 1/100 plant sap dilution was tested to detect cross-reaction between antigens and antibodies.

DISCUSSION

We have evaluated and compared PAS-ELISA, ACP-ELISA, and indirect ELISA kit for detection of CMV, PRSV-W, SqMV, WMV and ZYMV in infected and healthy *Nicotiana tabacum cv. Xanthi*-nc and *Cucurbita pepo* saps.

Our result showed (Tables 1, 2, 3) that CMV-C antiserum to cucumber mosaic virus did not work well in both PAS-ELISA and ACP-ELISA. This may result from quality of antiserum. CMV reacted with CMV-9 and CMV antisera in PAS-ELISA, ACP-ELISA, and indirect ELISA kit, but PAS-ELISA gave the strongest absorbance values in three assays. This results indicated that CMV can be detected by all three assays however, Indirect ELISA kit assay may be recommended to detect CMV because of its specificity and easiness.

Squash mosaic virus specifically and strongly reacted with SqMV antiserum in PAS-ELISA, but not ACP-ELISA and indirect ELISA formats. This results demonstrated that ACP-ELISA and indirect ELISA formats were not suitable for detecting this virus. It has been reported that buffers used during the extraction of the virus can greatly affect results (Huth et al., 1984; Carroll et al., 1995). Carroll et al. (1995) compared carbonate buffer and citrate buffer in indirect ELISA and found that the citrate buffer was better in detection of antigens at higher dilutions. In our study coating buffer (pH 9.6) and extraction buffer (pH 9.8) were used to coat plates in ACP-ELISA and indirect ELISA kit, respectively. Since the pH of the buffers were very high in both assays, the buffers used might have role in interfering with reactions between antigen and antibody or canceling binding between antigen and solid phase either by disrupting coat protein of virus or by changing charge and conformation of viral coat protein.

PRSV-W and WMV antisera cross-reacted with all three potyviruses (PRSV-W, WMV and ZYMV). Because these viruses contains very similar gene sequences of coat proteins (Yeh et al., 1992) it is expected to have some cross-reaction between antisera and these viruses. ZYMV antiserum was more specific than both PRSV-W and WMV antisera in all three assays. This result indicated that the quality of ZYMV antiserum was better than other two antiserum.

Protein- A ELISA is very sensitive and specific for detecting plant viruses. This method detects a broader spectrum of viral antigens than DAS-ELISA. The assay does not need conjugation to homologous antibodies and has ability to quantify viral antigens (Edwards and Cooper, 1985); however, PAS-ELISA is labor-intensive in sample preparation, containing more steps and using more time and reagents (Converse and Martin, 1991). For these reasons, it may not be recommended for large number of sample detection.

COMPARISON OF THE FORMATS OF THREE ELISA (PAS-ELISA, ACP-ELISA, INDIRECT ELISA KIT) AND REAGENTS FOR DETECTION OF SOME VIRUSES INFECTING CUCURBITS

Our results has indicated that indirect ELISA kit can be very useful in routine virus diagnoses and field surveys. The assay can easily be performed in very short time (5 hr.). It is not labor -extensive and uses less reagents. In literature, it has been reported that coating plates with direct antigen ELISA formats are not as specific as DAS-ELISA or PAS-ELISA (Lommel et al., 1982, Edwards and Cooper, 1985, Hobbs et al., 1987). For this reason, it may not be recommended to determine the serological variability among isolates and strains and quantifying viral antigens.

ÖZET

KABAKGİL VİRUSLARININ TEŞHİSİNDE KULLANILAN ELISA TİPLERİNİN (PAS-ELİSA, ACP-ELİSA, INDİREKT ELİSA) ve BAZI KİMYASALLARIN ETKİNLİKLERİNİN KARŞILAŞTIRILMASI

Bu çalışmada Hıyar Mozayık (CMV), Papaya Halkalı Leke Virus tip-W (PRSV-W), Kabak Mozayık (SqMV), Karpuz Mozayık (WMV) ve Zucchini Sarı Mozayık (ZYMV) viruslarının teşhisinde Protein-A ELISA (PAS-ELISA), Antijen Kaplı ELISA tabağı ve ticari olarak mevcut olan Indirekt ELISA yöntemleri ve bazı kimyasallar karşılaştırılarak en uygun metod belirlenmiştir. Hıyar mozayık virusu her üç ELISA metodu ile de teşhis edilmiştir. Fakat bu virusun tesbit edilmesinde PAS-ELISA ve Indirekt ELISA yöntemleri daha iyi bir performans göstermiştir. Kabak mozaik virusu sadece PAS-ELISA yöntemi ile tespit edilmiş, diğer iki metot bu virusun teşhisinde istenen sonucu verememiştir. Üç potyvirus PRSV-W, WMV, ZYMV kendilerine ait antiserumlar ile saptanabilmiş ancak her üç ELISA yönteminde de antiserumlar ve viruslar arasında çapraz reaksiyonlar tesbit edilmiştir. Bu çalışmadan çıkan sonuçlara göre, eğer rutin virus teşhisi ve arazi survey çalışmaları yapılacaksa indirekt ELISA (SqMV hariç) en uygun yöntem olarak tespit edilmiştir. Çünkü bu metot kısa zaman (5 saat) içerisinde, az emek sarf ederek ve daha az kimyasal kullanarak yapılabilmektedir. Eğer izolatlar ve ırklar arasında serolojik farklılıklar ve virus antijen yoğunluklarının belirlenmesi amaçlanıyorsa PAS-ELISA yönteminin daha uygun bir metot olduğu söylenebilir. Çünkü bu metot uzun zaman ve daha fazla iş gücü gerektirmesine rağmen bu virusların teşhisinde daha hassas ve spesifik sonuçlar verdiği saptanmıştır.

Anahtar kelimeler: Virus, PAS-ELISA, ACP-ELISA, Indirect ELISA, CMV, ZYMV, WMV, PRSV-W, SqMV, ELISA formatları

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J. Turk. Phytopath., Vol. 29, No. 2-3, 133-149, 2000 ISSN 0378 - 8024

Biological Control of *Fusarium oxysporum f. sp. melonis* by the Formulations of Fluorescent Pseudomonads

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ABSTRACT

A total of 126 fluorescent pseudomonad (FP) strains were obtained by isolations from the rhizoplane of more than 300 healthy cucurbitaceous plants. Of the FP strains, 64 produced siderophore and showed inhibitive effect against *Fusarium oxysporum f. sp. melonis* race 1,2 (FOM) *in vitro*. The suspensions of 28 strains, which were inhibitive more than 50% *in vitro* against FOM, were tested by seed bacterization for their reducing effect on Fusarial wilt severity in muskmelon in pots. Six of the FP strains were the most effective against FOM by giving 70.93% to 84.11% reduction in disease severity, as compared with the pathogen-alone treatment in pot tests. Cell suspensions of these strains were tested by seed bacterization for their ability to control Fusarium wilt of muskmelon under the field conditions in two consecutive years. In the field trials, three strains of *Pseudomonas putida*, i.e. 30, 109, and 180, significantly reduced the severity of FOM by 80%, 82%, and 84%, respectively, compared with the control plots without the FP strains. These promising strains of *P. putida* were selected for the development of various wettable powder formulations. In the experiment measuring long-term survival of *P. putida* strains, the talc-based formulations were found to be the best, yielding populations of 10^{9-10} CFU/g after 180 days of storage at 10°C. The bacterial population in kaolinite-based formulations has declined to 10^{8-9} CFU after 180 days of storage at 10°C. However, bacterial population in whey-based formulations dropped sharply after 60 days of storage at 10°C. Storage of the bioformulations at room temperature (24°C) caused a rapid decline in the population level of FPs. Talc-based formulations of the three strains of *P. putida* were tested for their ability to suppress the development of FOM under in-vivo conditions. In the pot tests, talc-based bioformulations of *P. putida* strains which were applied to the seeds of muskmelon reduced the percentage of wilt severity at the range of 76.13% to 87.65%, and were found to be more effective than Benomyl, used as reference fungicide.

Key words: Biological control, *Fusarium oxysporum* f. sp. *melonis*, *Pseudomonas putida, P. fluorescens,* bioformulation

BIOLOGICAL CONTROL OF *Fusarium oxysporum f. sp. melonis* BY THE FORMULATIONS OF FLUORESCENT PSEUDOMONADS

INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum f. sp. melonis* race 1,2 (FOM) is a very serious disease in muskmelon growing areas of Turkey (YILDIZ, 1977, TEZCAN ve YILDIZ, 1991). The pathogen is a soil-borne fungus and drenching with fungicides is very expensive and impractical for the extensive area. Although the use of Fusarium-resistant muskmelon cultivars can provide some degree of control of the disease, there is no commercially acceptable cultivar with adequate resistance to race 1,2 of FOM (SHERF and MAC-NAB, 1986, BLANCARD et al., 1991,).

Biological control of Fusarium wilts of numerous crops by application of antagonistic fungi and bacteria isolated from suppressive soils has been accomplished during recent years all over the world (TURHAN, 1981, SCHER and BAKER, 1982, PARK et al., 1988, FUCHS and DEFAGO, 1990, LEMANCEAU et al., 1992, VIDHYASEKARAN and MUTHAMILAN, 1995, LARKIN et al., 1996, LARKIN and FRAVEL, 1998, DUIJFF et al., 1999). Among these antagonists, non-pathogenic *Fusarium oxysporum* has received much attention and has been used to reduce Fusarium wilt diseases of various crops (PARK et al., 1988, MANDEEL and BAKER, 1991, ALABOUVETTE et al., 1993, LEMANCEAU et al., 1993, LARKIN et al., 1996). In additon, several biocontrol bacteria, including *Pseudomonas* spp., *Paenibacillus* sp., and *Streptomyces* sp., have been used to control Fusarium wilt diseases (TURHAN, 1981, SCHER and BAKER, 1982, VAN PEER et al., 1991, LIU et al., 1995, RAAJMAKERS et al., 1995). Among these bacterial antagonists, different strains of *Pseudomonas putida* and *P. fluorescens* have received much attention for controlling the Fusarium wilt (SCHER and BAKER, 1982, WELLER, 1988, LIU et al., 1995, VIDHYASEKARAN and MUTHAMILAN,1995). Several mechanisms have been proposed to be involved in the suppression of *Fusarium oxysporum* by fluorescent pseudomonads (FP): (i) The suppression may be due to competition for iron (KLOEPPER et al., 1980, SCHER and BAKER, 1982), (ii) these bacteria also produce several antibiotics (WELLER, 1988, ALABOUVETTE et al., 1996), and (iii) the induced systemic resistance may be a mechanism of biological control of Fusarium wilts by FPs (LEEMAN et al., 1995, LARKIN et al., 1996, VAN LOON et al., 1998).

Application of FPs for control of soil-borne diseases depends on development of their commercial formulations in which the bacteria can survive for a considerable length of time, and assessment of *in vivo* efficacy of these formulations for disease control. The objectives of this research were to isolate effective FP strains from the rhizoplane of Cucurbitaceous plants, screen them for their biocontrol capabilities *in vitro*, and test the FP strains producing siderophore in both pot and field trials for their antagonistic effect, and finally, to develop various formulations of the FP strains, to control Fusarium wilt of muskmelon under *in vivo* conditions.

MATERIALS and METHODS

Isolation of fluorescent pseudomonads. In this study, rhizoplane colonizing FP strains were isolated from the healthy root samples collected from more than 300 fields of muskmelon, watermelon and cucumber in Aegean Region. After washing the excised roots to remove adhering soil, root segments of approximately 1 g were added to 100 ml of sterile destilled water in flasks and shaken by a rotary shaker at 150 rpm for 30 min (GEELS and SCHIPPERS, 1983). FPs were isolated using King's medium B (KING et al., 1954) with antibiotics (100 ppm cychloheximide, 50 ppm ampicilin, 12.5 ppm chloramphenicol). The FPs were selected under UV light (366 nm) from developing bacterial colonies.

***In vitro* antibiosis assays**. The strains of FP isolated from the rhizoplane of cucurbitaceous plants were placed on four different points at equal distance from edge of the petri dish (9 cm ∅), containing King's Medium-B, incubated for 48 h to allow development of bacterial colonies. A conidial suspension of FOM (10^5 conidia/ml) was sprayed onto the bacterial colonies. After incubation for 5 days at 24°C, the inhibition zones developed between the colonies of FPs and FOM were evaluated by 0-5 scale (GEELS and SCHIPPERS, 1983). *In vitro* tests were conducted with 3 replications.

Determination of siderophore production. The antagonistic FP strains to FOM were planted on four different points of petri dishes containing 80 µM FeCl3/L added King's Medium-B. After 48 hours, the dishes were sprayed with conidial suspension of FOM (10^5 conidia/ml). If no inhibition zone developed between the colonies of FP and FOM within 5 days, it was considered that the antagonism may be due to the production of siderophore (GEELS and SCHIPPERS, 1983, ELAD and BAKER, 1985). The siderophore tests were also conducted with 3 replications.

Pot tests. In *in vitro* screening tests, the FP strains which inhibit the colony development of FOM by more than 50%, were selected for the pot tests. The antagonistic effect of FP strains were tested by seed-bacterization in pots. The soil used in the pot tests held: pH:7.46; lime: 5.33% (rich); sand: 66.88%; silt or allivium: 25-28%; kaolinite: 7.84%; and iron: 3.7 ppm (poor). KNO_3 (200 ppm) was added to the soil in order to enhance the antagonistic effect of FP strains (BORA et al., 1992). The soil disinfected with formaline solution was inoculated with the culture of FOM, developed on sterilized wheat grains for 7-10 days, at the rate of 4 g/L soil (REDDY and PATRICK,1990). The pots (20 cm ∅) were filled with disinfected and inoculated soil. Muskmelon seeds (*Cucumis melo* cv. Kırkağaç) were treated with the suspension of antagonistic bacteria at the concentration of 10^9 CFU per ml (50 µl/seed), prepared in 1% CMC solution with 24 h old culture of the bacteria, for a period of 4 hours. Each treatment consisted of five replicate pots with three to four plants in each. In case of

positive control treatment, the pots containing soil inoculated with FOM were sown by the seeds uninoculated with the antagonistic bacteria. Plants were grown in a climatized room on a cycle of 14-h dark and 10-h light at 24°C for 30 days.

Field trials. The strains of siderophore producing fluorescent pseudomonads (SPFP), which showed high efficacy in *in vitro* conditions and reduced the severity of Fusarium wilt by 70 % in pot tests, as compared to the pathogen-alone, were selected for further field trials. Field trials were repeated for two years (1994 and 1995) and conducted in two different towns of İzmir: Menemen and Bornova. Seed-bacterization and soil inoculation with FOM were done by using same techniques. The muskmelon seedlings grown in polyethylene tubes for 20 to 25 days were transplanted to the plots in fields. Trials were carried out using complate randomized block design with 5 replications. Each plot consisted of three rows with 5 plants in each row.

Evaluation of pot tests and field trials. Four weeks after sowing in pots, disease severity was recorded by using 0-4 scale (SUNG and HUANG, 1984). In the field trials, observations were made beginning from the appearence of first visible symptoms until the harvesting time, periodically and the same scale was used. Disease reduction was calculated as the percentage reduction of disease compare to the pathogen-alone control. Data from the last observation date were subjected to variance analysis, and the means were compared using the least significant difference test. Significance was evaluated at $P = 0.05$ for all tests. All data expressed as percentages were arcsine-transformed before analysis.

Identification of antagonistic fluorescent pseudomonad strains. The bacterial strains which showed inhibition against FOM under field conditions were identified by various physiological and biochemical tests specific for FPs (HILDEBRAND and SCHROTH, 1971, PALLERONI, 1984).

Development of bioformulations of the bacterial agents. FP strains found to be effective against FOM under field conditions were grown on liquid King's Medium-B for 24 hours as a shake culture incubating in a rotary shaker at 150 rpm at room temperature (24±1°C). Bacterial suspensions were then centrifuged for 20 min at 6,000 rpm. Pellets were resuspended in 0.1 M $MgSO_4$ in a ratio 1:1 (w/v). Bacerial suspensions in 0.1 M $MgSO_4$ were mixed with a 10% (v/v) glycerole, which was used as a bacterial preservative. Then, this suspension was mixed with an equal volume of autoclaved 1.5% Na-Alginate (BASHAN, 1986, DIGAT, 1990). Bacteria – Na Alginate mixture was mixed with sterilized carriers such as talc, kaolinite, and whey at the ratio approximately four times the volume of the bacteria – Na alginate mixture (1:4 v/v) (KLOEPPER and SCHROTH, 1981). As a wetting agent 7% Ca-Lignine was added to the mixture. The resulting mixture was thinly spread over a glass sheet and air-dried in a laminar air- flow

cabine at 24°C for 1 h to form a slightly moistened powder (15% moisture content). After drying, bacterial formulations were powdered in a Waring Blendor and stored in glass bottles with lids as small volumes (10g) (SUSLOW and SCHROTH, 1982, CONNICK, 1988, PETROLINI et al., 1998).

Survival of FP strains in different formulations. Bacterial formulations were stored under two different conditions: at 24°C for 120 days and at 10°C for 180 days. The antagonistic bacteria in each formulation were monitored *in vitro* with respect to their shelf-life and viability under various storage conditions for this period. Survival of bacterial population in the formulations was assessed at 60 days intervals using King's Medium-B by dilution plate method (VIDHYASEKARAN and MUTHAMILAN, 1995). There were three replications for each analysis.

Biological control assays with antagonist formulations. The talc-based formulations of FP strains, survived at 10°C for a period of 180 days, were tested for their effectiveness to control the Fusarium wilt of muskmelon in pots. FOM was grown in potato dextrose liquid medium as shake culture incubating in a rotary shaker with 150 rpm at 24°C. After 7 days, cultures were filtered through cheese cloth to remove mycelial mass. Microconidial densities in the filtrate were determined by a haemocytometer and adjusted by dilution to 10^5 conidia/ml. Each pot (20 cm ∅) was filled with 1 Kg of sterilized soil inoculated with 100 ml conidial suspension of FOM (LARKIN and FRAVEL, 1998). After 24 h, the bacterial formulations were suspended in destilled water to the end concentration of 10^9 CFU/ml. Muskmelon seeds were treated with the bioformulation suspensions (10^9 CFU/ml) at 50 µl per seed for 2 h. Treated seeds were sown in pots containing the soil inoculated with FOM. To assess the efficacy of bacterial formulations against FOM, 0.15% Benomyl was used as reference preparation. Each treatment consisted of ten replicate pots with four plants in each. The pots were kept in climatized room on a cycle of 10-h light and 14-h dark duration at 24°C for 48 days. Percentage of wilt severity was evaluated by 0-4 scale, as described before.

RESULTS

***In vitro* tests**. A total of 126 FP isolates were obtained from the rhizoplane of more than 300 healthy muskmelon, watermelon, and cucumber plants in 2 years. Of the 126 FP strains, 64 showed inhibitory effect against FOM, at different levels when *in vitro* antagonism tests were evaluated by 0-5 scale (Table 1). It has been observed that all of the FP Strains which inhibited FOM in *in vitro* tests, were capable to produce siderophore.

Bioefficacy of selected fluorescent pseudomonad strains on the severity of Fusarium wilt of muskmelon. Of the 64 FP strains tested, 28 inhibiting the growth of

Table 1. Distribution of the FP strains which were antagonistic to FOM in *in vitro* tests

Categories of inhibition (0-5)	Efficacy (%)	Number of the Effective FP Strains
0.0 – 0.9	0 – 20	4
1.0 – 2.0	21 – 40	10
2.1 – 3.0	41 - 60	34
3.1 – 4.0	61 – 80	7
4.1 – 5.0	81 - 100	9
TOTAL		64

FOM by more than 50% were selected and tested for their ability to control Fusarium wilt of muskmelon by seed bacterization in the pot test. Of the 28 FP strains, 10 were from healthy muskmelon, 12 watermelon, and 6 cucumber roots (Table 2).

Approximately 61% of the FP strains tested, significantly reduced Fusarium wilt of muskmelon by 55.64% to 84.11% relative to the pathogen-alone (Table 2). These strains inhibited the colonial growth of FOM at a rate more than 50% in in-vitro assay (Table 2). Six of them, viz., 2, 30, 109, 180, 217, and 235, were the most effective against FOM giving 83.20%, 77.66%, 77.23%, 84.11%, 75.59%, and 70.93% reduction, respectively. The first three strains were tested for their ability to control the Fusarium wilt of muskmelon by seed bacterization under field conditions in 1994. The second three strains were similarily tested in 1995.

In the first trial conducted in 1994, early symptoms were observed 10 days after transplanting the plants. Three FP strains,viz., 2, 30, and 109, significantly reduced Fusarium wilt of muskmelon for a period of 60 days after transplanting by 65%, 80%, and 82%, respectively (Fig 1). But disease severity increased along with the increase in the senescence of the plants. At the final observation date, FP strains provided significant reduction of the disease severity, but generally did not reduce the disease at the levels greater than 37-47 % (Fig 1).

During the second field trial, conducted in 1995, the disease was more effectively controlled by FP strains by seed treatments than the first field trial conducted in 1994. Even 93 days after transplanting, three FP strains, viz., 180, 217, and 235 significantly reduced the severity of Fusarium wilt by 84%, 68%, and 66%, respectively (Fig 1).

Using the conventional technics based on the groupings of Hildebrand and Schroth (1971) and Palleroni (1984), 5 of the 6 effective FP strains were identified as *Pseudomonas putida*, and one (strain 217) as *P. fluorescens*. None of the bacterial strains caused soft rot or a hypersensitive reaction on tobacco. Three promising strains of *P. putida*, 30, 109, and 180, were selected for the development of bioformulations.

Table 2. Effectiveness of selected FP strains in *in vitro* and in controlling Fusarium wilt of muskmelon in pot test.

Isolate Number	% Wilt Severity[x]	% Reduction in severity[y]	*in vitro* efficacy %[z]	Origin
155	58.65 a	---	85.00	muskmelon
65	52.12 ab	---	95.00	watermelon
Pathogen-only	51.60 ab	---	---	---
287	46.00 abc	10.86	75.00	cucumber
28	45.16 bc	12.48	65.00	watermelon
41/1	40.27 bcd	21.96	78.60	watermelon
20	38.94 bcd	24.54	82.00	watermelon
14	37.40 cd	27.52	60.00	watermelon
276	36.00 cd	30.24	60.00	cucumber
63	32.78 cde	36.48	100.00	watermelon
282	30.00 def	41.86	53.20	cucumber
67	28.91 defg	43.98	97.40	muskmelon
38/1	22.88 efgh	55.66	96.40	watermelon
62	22.87 efgh	55.68	95.00	muskmelon
72	22.89 efgh	55.64	82.50	watermelon
221	23.00 efghi	55.43	68.20	muskmelon
252	21.30 efghij	58.72	60.00	muskmelon
281	20.00 fghij	61.24	53.20	cucumber
33	19.33 fghijk	62.54	90.00	muskmelon
274	18.00 ghijkl	65.12	50.00	cucumber
259	18.00 hijkl	65.12	60.00	watermelon
235	15.00 hijklm	70.93	65.00	muskmelon
273	14.50 hijklm	71.90	55.00	cucumber
256	12.56 ijklmn	75.66	50.00	watermelon
30	11.53 jklmn	77.66	70.00	muskmelon
217	12.60 klmn	75.59	60.00	muskmelon
109	11.75 lmn	77.23	60.00	muskmelon
2	8.67 mn	83.20	66.66	watermelon
180	8.20 n	84.11	50.00	watermelon

[x] Means followed by the same letter are not significantly different (P>0.05) according to Fisher's protected least significant difference (LSD= 7.843) test.

[y] Disease reduction (% reduction) represents the percentage reduction of disease relative to the pathogen-only control.

[z] Antagonistic effect was measured by 0-5 scale. Mean value of 3 plates with 4 measurements in each.

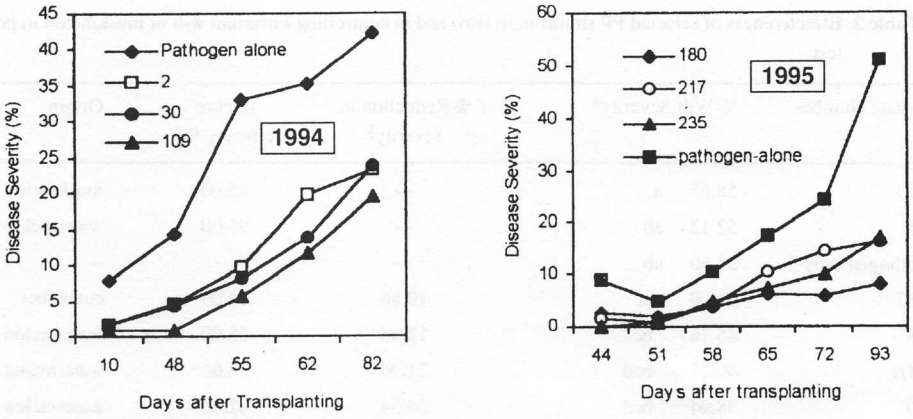

Figure 1. Development of Fusarium wilt of muskmelon plants as affected by seed treatment with some fluorescent pseudomonad strains in the field tests conducted at two consecutive years (1994 and 1995).

Survival of *P. putida* strains in various formulations. The initial high populations of *P. putida* strains in the wettable powder formulations were not necessarily sustained during the storage at 10°C. There was a subsequent decline in the population; 60 days after incubation the bacterial population decreased from 10^{11-14} to 10^{9-10} CFU per gram in talc-based, kaolinite-based, and whey-based formulations (Fig 2). In the experiment measuring long-term survival of *P. putida* strains, the talc-based formulations were found to be the best, yielding populations of 10^{9-10} CFU per gram after 180 days of storage at 10°C. The bacterial population in kaolinite-based formulations has declined to 10^{8-9} CFU after 180 days of storage at 10°C. However, bacterial population in whey-based formulations dropped sharply after 60 days of storage at 10°C (Fig 2). Storage of the bioformulations at room temperature (24°C) caused a rapid decline in the population level of FPs (Table 3). After storage for 60 days at room temperature, FP populations in talc-based, whey-based, and kaolinite-based formulations decreased approximately 50% as compared with the initial populations (Table 3). By aging, some changes have been observed in the colonial characteristics and pigmentation of the FP strains.

Effectiveness of the bacterial bioformulations in pot tests. Talc-based formulations of the three strains of *P. putida* were applied to muskmelon seeds. According to the observations which were made 48 days after sowing, talc-based formulations of *P. putida* strains, viz., 30, 109, and 180, significantly reduced the severity of Fusarium wilt by 87.65%, 77.77%, and 76.13%, respectively, compared with 50.62% severity of wilt in untreated control plants (Fig 3). All the bioformulations provided better disease control (P=0.05) than Benomyl (Fig 3).

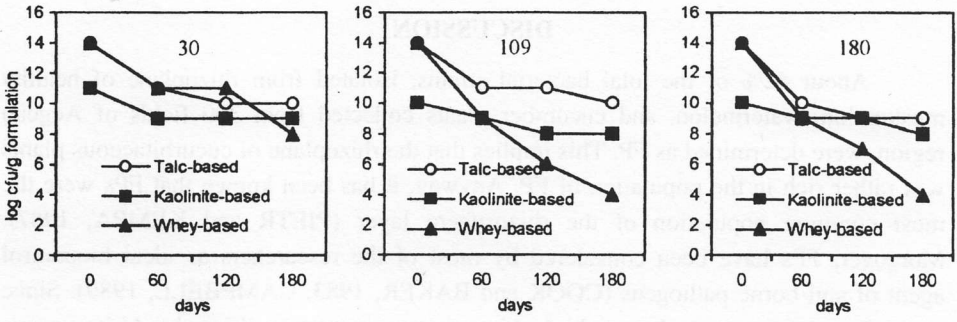

Figure 2. Survival of *P. putida* strains of 30, 109, and 180 in various formulations stored at 10°C for 180 days.

Table 3. Survival of *P. putida* strains of 30, 109, and 180 in various formulations stored at 24°C for 120 days.

Isolate Number	Carrier Formulation	Moisture Content of Formulation (%)	Population (CFU per g dried formulation) at various days of storage		
			0	60	120
30	Talc-based	15.65	$2.4x10^{14}$	$1.8x10^{11}$	$1x10^{3}$
	Kaolinite-based	14.43	$6.3x10^{11}$	$2.6x10^{7}$	$1x10^{3}$
	Whey-based	13.00	$6.5x^{15}$	$1.5x10^{9}$	--*
109	Talc-based	15.70	$4.7x10^{13}$	$6.8x10^{8}$	$2x10^{3}$
	Kaolinite-based	14.90	$3.5x10^{10}$	$2.6x10^{9}$	$1x10^{3}$
	Whey-based	15.80	$2.4x10^{13}$	$2.9x10^{7}$	--
180	Talc-based	15.20	$5x10^{13}$	$1.5x10^{7}$	--
	Kaolinite-based	14.00	$2.7x10^{10}$	$2.3x10^{7}$	--
	Whey-based	15.33	$6.7x10^{13}$	$7.9x10^{7}$	--

* No visible growth

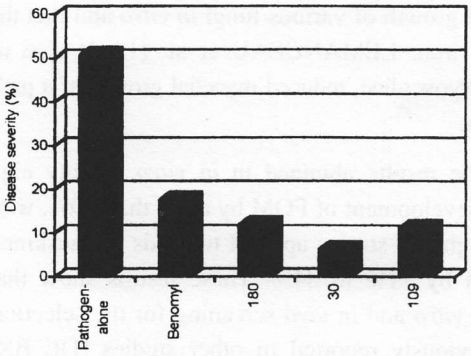

Figure 3. Effect of talc-based formulations of *P. putida* strains on suppression of Fusarium wilt of muskmelon. Talc-based formulations were prepared by diluting with distilled water to 10^9 CFU/ml, and each of them were applied at 50 µl/seed for 2 h. Data are from a single representative test and the means of ten replicates per treatment.

DISCUSSION

About 42% of the total bacterial strains, isolated from rhizoplane of healthy muskmelon, watermelon, and cucumber plants collected from 300 fields of Aegean region, were determined as FP. This implies that the rhizoplane of cucurbitaceous plants was rather rich in the population of FP. Anyway, it has been known that FPs were the most common population of the rhizosphere layer (PIETR and KEMPA, 1987). Moreover, FPs have been considered by most of the researchers as ideal biocontrol agent of soil-borne pathogens (COOK and BAKER, 1983, CAMPBELL, 1989). Since many *Pseudomonas* spp. have a high rhizosphere competence, there should be intense competition for Fe^{3+} at the rhizoplane when the *Pseudomonas* produces siderophores. Therefore, Fe^{3+} is bound in such a way that is unavailable to Fusarium. Thus, application of FPs to soils has induced suppressiveness (SCHER and BAKER, 1982).

In this research, approximately 78% of the FP strains, inhibited the colony development of FOM by 40 to 100% in *in vitro* (Table 1, 2). It has been observed that all of the FP strains which inhibited FOM in *in vitro* tests were able to produce siderophore (Table 2). FPs did not produce fluorescent siderophores on agar medium containinig (80µM/L) Fe^{3+}. Presence of $FeCl_3$ in the agar medium inhibited production of fluorescent siderophores. These results can be explained with that the soils of Aegean Region are more alkaline in nature and deficient in iron. It has been suggested that competition for iron (Fe^{3+}) was responsible for the suppressiveness in these systems since Fe appeared to be necessary for germ-tube elongation of *F. oxysporum* microconidia (SCHER and BAKER, 1982). Fe EDDHA and siderophores produced by *P. putida* are considered to have higher Fe stability constants than siderophores shown to be produced by various pathogenic fusaria (SCHER and BAKER, 1982). Our results are confirmed by MISAGHI et al. (1982), by reporting that a partially purified siderophore of *Pseudomonas* inhibited growth of various fungi *in vitro* and that the effect was nullified by addition of excess iron. LEMANCEAU et al. (1993) also reported that purified siderophore, named as Pyoverdine, reduced mycelial growth of a pathogenic *F. oxysporum* due to iron competition.

According to the results obtained in *in vitro*, twenty eight FP strains, which inhibited the colonial development of FOM by more than 50%, were selected for the pot tests. Of the twenty eight FP strains applied to seeds of muskmelon, 6 were the most effective against FOM by 71% to 84%. These results show that there is a positive correlation between *in vitro* and *in vivo* screening for the selection of these biological control agents, as previously reported in other studies (DE BRUYNE et al., 1990, REDDY et al., 1990).

When three *P. putida* strains i.e. 30, 109, and 180, which were found to be the most effective in the pot tests, were applied to the seeds of muskmelon, the severity of

wilt caused by FOM were reduced by 80% to 84% under field conditions for 60 days (Fig 1). These findings have been found in accordance with the results of pot tests (Fig. 1, Table 2). FP strains applied to seeds can colonize the rhizoplane readily at the beginning of the cropping period before the colonization by the pathogen, because ecological interactions between pathogen-host-antagonist and resident microflora are less complicated (SINGH et al., 1999). On the last observation date (approximately 90 days after transplanting), *P. putida* strain 180 significantly reduced Fusarium wilt of muskmelon by 84%, whereas the efficacy of *P. putida* strains 30, and 109 gradually decreased to 40%. Suppression of the disease for such a long period, despite the expectation of a low biocontrol effect and diminishing in FP population due to high atmospheric temperature and drought, especially during July, cannot be explained via only the inhibitory effect of siderophore to the spore germination. This situation can be explained by induction of resistance in the host plant by biocontrol agents is another mode of action that can suppress Fusarium wilt, besides microbial competition (FUCHS and DEFAGO, 1990, VAN LOON et al., 1998). Previous studies have demonstrated that non-pathogenic *F. oxysporum* Fo47, *P. putida* WCS358, and the other rhizobacteria can induce systemic resistance in plants against Fusarium wilt (VAN LOON et al., 1998, DUIJFF et al., 1999). In addition, siderophores and, more specifically, pyoverdines have been shown to play a role in the induction of resistance in the host plant against different diseases (MAURHOFER et al., 1994, LIU et al., 1995).

It has been suggested that microorganisms isolated from the root or rhizosphere of a specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other plant species (COOK, 1993). However, some results of investigation have claimed the opposite of this hypothesis. Some researchers have suggested that the mechanism of action was not host-specific (SPURR, 1981, LARKIN and FRAVEL, 1998). Among the three strains of *P. putida* successfully inhibiting Fusarium wilt of muskmelon under field conditions, two (30 and 109) were isolated from the rhizoplane of muskmelon, whereas one (180) was isolated from the rhizoplane of watermelon. Although the two host plants don't belong to the same species, but these are the members of the same Family.

Seed treatment with cell suspensions of *P. putida* has been found to be effective in controlling several diseases (KLOEPPER and SCHROTH, 1981, PARKE et al., 1991, VIDHYASEKARAN and MUTHAMILAN, 1995). For commercial exploitation, this methodology will be impractical due to difficulty in handling, transportation, and storage (DIGAT, 1990). The commercial use of fluorescent pseudomonads requires inoculum that retains a high cell viability and can easily be transported, and applied to seed (KLOEPPER and SCHROTH, 1981, STEINBERG et al., 1997). The addition of an

inert but hydrophilic protective carrier, such as kaolinite, peat, talc or cellulose is necessary to obtain good efficiency (SUSLOW and SCHROTH, 1982). However, one of the main problems is that microorganisms, before becoming active, have to be rehydrated, which might cause a loss in viability due to osmotic shock. Gelled formulations, using polymers such as polyacrylamide polysaccharides such as Xanthan (KLOEPPER and SCHROTH, 1981, JUNG et al., 1982), or alternatively alginate (BASHAN, 1986, DIGAT, 1990) solve this problem. We used 1.5 % Na-Alginate, and observed that alginate seemed to be a well-protective medium for bacterial survival. In the present study, we have shown that *P. putida* strains could survive well in talc-based and kaolinite-based formulations stored at 10°C for a period of 180 days (Fig 2). FPs have been reported to survive in certain dry formulations (CONNICK, 1988). It has been reported that the populations of FPs did not decrease in the talc mixture with 20% xanthane gum after storage for 2 months at 4°C (KLOEPPER and SCHROTH, 1981), and in the vermiculite-based dried formulation after storage for 6 months at 4°C (CONNICK, 1988). Our findings are also confirmed by these results. However, it has been observed that the shelf-life of whey-based formulations stored at 10°C were less than 180 days, and the population rapidly declined as of 120 days (Fig 2). The reason of failure of whey-based formulations can be explained by that the pH of whey is lower than 5.0, and rich in carbohydrate.

One of the key factors in survival is the preservation of residual hygrometry of about 15% in dried formulation (DIGAT, 1990). The moisture content in the bioformulations developed was also measured as 15% after drying (Table 3). Our results are also confirmed by DIGAT (1990).

Seed bacterization with talc-based formulations of *P. putida* strains such as 30, 109, and 180, significantly reduced the severity of Fusarium wilt of muskmelon in pot tests by 76 to 87%, compared with the control (Fig 3). The suppressiveness of the talc-based formulations in a dried powder remained significantly active after 180 days of storage at 10°C. It has been determined that the severity of Fusarium wilt was inhibited by 77% to 84% in pot tests (Table 2) when the cell suspensions of the same strains of *P. putida* were applied to seeds of muskmelon. The effectiveness of the cell suspensions of same strains under field conditions was determined to be between 80% and 84%.

Results obtained in the present study revealed that talc-based formulations of *P. putida* strains can be used effectively as seed treatments for the control of Fusarium wilt of muskmelon. Further studies are in progress to increase the shelf-life of *P. putida* strains in talc-based formulations using some adjuvants and stabilizators, and to determine the efficacy against FOM as seed bacterization treatment under field conditions.

ÖZET

Fusarium oxysporum f.sp. melonis'in **FLORESENT PSEUDOMONAS FORMULASYONLARIYLA BİYOLOJİK KONTROLÜ**

Cucurbitaceae familyasına ait 300 kadar sağlıklı bitkinin kök yüzeyinden yapılan izolasyonlarda, toplam olarak, 126 fluoresent pseudomonas (FP) izolatı elde edilmiştir. Bunlardan 64 tanesi siderofor üretmiş ve *in vitro*'da *Fusarium oxysporum f.sp. melonis* ırk1,2 (FOM)'de koloni gelişimini engelleyici etki göstermiştir. *In vitro*'da FOM'u %50'den daha çok engelleyen 28 izolatın süspansiyonu, saksı denemelerinde, tohum bakterizasyonu yoluyla sisteme sokulduğunda kavunda Fusarium solgunluğunun şiddetini azaltmıştır. Etkili FP izolatlarından 6 tanesinin, solgunluk şiddetini %70.93-%84.11 arasında engellediği saptanmıştır. Bu izolatların hücre süspansiyonlarıyla tarlada 2 yıl üstüste tohum bakterizasyonu yoluyla denemeler yapılmıştır. Bu tarla denemelerinde *Pseudomonas putida*'nın 30, 109 ve 180 numaralı izolatları Fusarium solgunluğunun şiddetini, kontrola göre, sırasıyla, %80, %82, ve %84 oranında azaltmıştır. *P. putida*'nın umutvar olan bu izolatları çeşitli ıslanabilir toz formulasyonları hazırlanmak üzere seçilmişlerdir. Bu strainlerle hazırlanan talk-katkılı ıslanabilir toz formulasyonlarda bakteri 10°C'de 180 gün süreyle 10^{9-10} CFU/g düzeyinde canlılığını korumuştur. Kil katkılı formulasyonlarda ise bu değer 10^{8-9} CFU/g düzeyine düşmüştür. Oysa ki, peyniraltı suyu ile hazırlanan biyoformulasyonlarda aynı sıcaklıkta canlı hücre sayısı daha henüz 60. günde hızla düşüş göstermiştir. Biyoformulasyonların oda sıcaklığında (24°C) saklanması durumunda FP populasyonundaki düşüş çok hızlı olmuştur. *P. putida*'nın talk katkılı bu 3 formulasyonu *in vivo* koşullarda FOM'u baskılama yetenekleri yönünden de denenmiştir. Saksılarda yapılan bu çalışmalarda kavun tohumlarına uygulanan talk-katkılı *P. putida* biyoformulasyonları solgunluk şiddetini %76.13-%87.65 düzeyinde azaltarak referans fungisit olan Benomyl'den çok daha yüksek etki göstermişlerdir.

Anahtar kelimeler: Biyolojik savaş, *Fusarium oxysporum* f. sp. *melonis*, *Pseudomonas putida, P. fluorescens*, biyoformulasyon

ACKNOWLEDGMENTS

This research was supported by a grant from the Scientific and Technical Research Council of Turkey (TÜBİTAK).

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