Phytoparasitica (2011) 39:285–292
DOI 10.1007/s12600-011-0163-7

Seed transmission of verticillium wilt of cotton

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Received: 5 November 2010 / Accepted: 16 April 2011 / Published online: 4 May 2011

Abstract Twenty-nine cotton genotypes with varying levels of susceptibility to *Verticillium dahliae* were grown in infested plots at Nazilli, Aydın, in 2008–2009. The highest level of disease incidence was recorded in cultivars 'BA-151', 'Celia', 'Çukurova-1518', 'Flaş' and 'Maraş 92', and averaged 85–95% for all genotypes in both years. The incidence of *V. dahliae* in seed averaged 29.8% for cv. Çukurova-1518, 27.6% for Flaş, 24.6% for cv. BA-151, 19.0% for cv. Celia and 16.2% for Maraş 92. Two hundred seeds from each genotype were planted, two seeds per pot, in a steam-pasteurized mixture of soil, peat, and sand. Pots were placed close to each other on a greenhouse bench to obtain a thick canopy. Typical disease symptoms appeared about 12–13 weeks after sowing. Maximum disease incidence values averaged 3.3% for Celia, 4.5% for Maraş 92, 8% for BA-151, 9% for Flaş and 9.5% for Çukurova-1518.

Keywords *Gossypium hirsutum* · Seed infection · Soilborne pathogen · Vascular wilt · *Verticillium dahliae*

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Introduction

In Turkey, about 547,000 ha of upland cotton (*Gossypium hirsutum* L.) is grown annually under irrigation in three main regions: Aegean, Mediterranean and southeastern Anatolia. Verticillium wilt, incited by the soil-inhabiting fungus *Verticillium dahliae* Kleb., is among the most serious diseases of cotton throughout Turkey, causing substantial economic losses (Göre 2007). The pathogen infects roots, causing foliar wilt and defoliation. Disease severity depends on several factors, including the cultivar used, the phenological stage of the plant, environmental conditions, inoculum density in soil, and the virulence of *V. dahliae* strains (Göre *et al.* 2009). It was first reported in Turkey in 1941 (İyriboz 1941), but was not identified as an important disease under field conditions until 1967 (Karaca *et al.* 1971). Since the mid 1990s, verticillium wilt has increased progressively in many fields, and an unusually high incidence of a severe wilt disease of cotton has been observed in Turkey (Göre *et al.* 2007). In 2004 epidemics of verticillium wilt occurred in the Aegean region of Turkey. A defoliating pathotype of

V. dahliae was isolated from the diseased plant and later found to be widespread throughout the region to the Mediterranean and southeastern Anatolia regions (Derviş *et al.* 2008; Göre 2007; Göre *et al.* 2007).

The pathogen is spread from field to field and from one geographic area to another by several means, including wind and water movement (Easton *et al.* 1969; Lindemann *et al.* 1982) and soil adhering to seedlings (Xiao and Subbarao 1998), farm equipment, animals, or humans (Howard 1985; Huang *et al.* 1986; Price 1976) in the form of conidia, microsclerotia or as mycelium in infected host tissue. Probably the greatest potential for long distance dissemination of *V. dahliae* is either by seed infected with mycelia or by seed contaminated with microsclerotia (Evans *et al.* 1966; Karaca *et al.* 1973; Sackston 1983). As many as 100 live microsclerotia have been found on a single seed that had been contaminated at harvest time. One microsclerotium is enough to initiate infection of a plant, and high populations of the fungus increase the severity of the disease. The cotton plant is susceptible to verticillium wilt at any stage of growth from seedling to mature plant (Wilhelm *et al.* 1966).

In early studies seed transmission was considered unlikely, given that *V. dahliae* could not be isolated from fungicide-treated and acid-delinted seeds (Shen 1985; Wilhelm *et al.* 1966). However, *V. dahliae* was detected in several countries on three continents (Bejarano-Alcázar *et al.* 1997; Elena 1999; Katan 2000; Korolev *et al.* 2008; Zhengjun *et al.* 1998) during a 15-year period, and the disease often appeared in previously uninfested fields. For this reason, seed transmission of *V. dahliae* is being reexamined as a potential means of disease spread.

Understanding the seed transmission of *V. dahliae* in cotton is important for proper disease management programs. This information is a prerequisite for proper prevention of disease through seed treatment, setting tolerance levels for variety release, in inspection of farmers' seed production schemes, quarantine, germplasm management and exchange, and optimization of storage conditions (Agarwal and Sinclair 1997).

Although Allen (1951) first suggested seed as a source *of V. dahliae* in cotton, there has been no detailed study of its occurrence on cotton seed from different regions or climates, or of its transmission from seed to growing seedlings. Therefore, the objectives of this study were to determine the level of seed infection occurring in cotton genotypes susceptible to *V. dahliae* when planted in infested field plots and to determine seed transmission of the disease taking place in the greenhouse by planting naturally contaminated seed under verticillium wilt-free conditions.

Materials and methods

Seed infections Field investigations were conducted in 2008 and 2009 in a selected field that was known to be naturally infested with the nondefoliating pathotypes of *V. dahliae* belonging to VCG 2B (Göre 2007). This field has been used for cotton breeding trials at Nazilli Cotton Research Institute every year since 1972. The inoculum density was 80 and 85 propagules per gram of soil in 2008 and 2009, respectively. The number of *V. dahliae* propagules was counted in the soil samples as described previously (Huisman and Ashworth 1974). Briefly, a representative portion of soil sample (250 g) from each year was then taken and air-dried for 4 weeks under ambient conditions. Subsequently, each sample was passed through a 0.8-mm sieve to remove organic debris and large particles, followed by mixing by hand. The *V. dahliae* inoculum's density was estimated by wet sieving using 20 petri dishes containing modified NP-10 selective medium (Kabir *et al.* 2004). Plates were incubated at 22–24°C in the dark for 14 days, after which soil residues were removed with tap water and *V. dahliae* colonies counted by using a dissecting microscope with transmitted light. Counts from the replications were combined for mean values and expressed as microsclerotia per gram dry soil.

Twenty-nine of the most commonly grown cotton cultivars in Turkey were planted in 2008 and 2009 to study the epidemiology of and resistance to verticillium wilt in cotton (Table 1). These cultivars comprised approximately 98% of cotton plantings in Turkey in 2007 (Göre *et al.* 2009). Treatments were applied in plots that were 0.7 m wide (in two rows of approximately 60 plants per row) by 12 m long in a randomized complete block design (with 2-m intervals between blocks) with four replicates (Erdoğan and Benlioğlu 2010). At the end of the growing season (about 165 days after planting), each harvested seed lot was assayed for *Verticillium* using a

Table 1 Maximum incidence of verticillium wilt in cotton plots in 2008 and 2009 and the incidence of infection with *Verticillium dahliae* in seed harvested from the plots

Genotype	Company[w]	2008		2009		Average	
		DI (%)[x]	Incidence of *V. dahliae* (%)[y]	DI (%)	Incidence of *V. dahliae* (%)	DI (%)	Incidence of *V. dahliae* (%)
Aksel	Özbuğday	81 defg[z]	16.7 klm	88 abc	17.4 hij	84.5	17.1
BA-119	Özbuğday	84 cd	22.8 efg	72 fghi	19.2 fghi	78.0	21.0
BA-151	Özbuğday	87 bc	24.7 def	92 a	24.4 cd	89.5	24.6
BA-308	Özbuğday	77 fghij	17.5 jklm	82 cde	14.4 jkl	79.5	16.0
BA-525	Özbuğday	81 defg	32.3 b	72 fghi	23.3 cde	76.5	27.8
BA-Gold	Özbuğday	80 defgh	16.7 klm	89 ab	12.9 kl	84.5	14.8
Candia	Bayer	75 hijk	21.1 ghi	78 def	22.1 defg	76.5	21.6
Carmen	Bayer	68 lmn	20.5 ghij	53 l	18.4 fghi	60.5	19.5
Celia	Bayer	85 bcd	17.6 ijklm	86 abc	20.3 efgh	85.5	19.0
Çukurova-1518	ÇARI	97 a	27.9 cd	92 a	31.6 b	94.5	29.8
DD-493	Monsanto	76 ghij	14.4 mn	87 abc	18.2 ghij	81.5	16.3
Delta Opal	Monsanto	72 jklm	18.1 hijkl	76 efgh	16.1 ijk	74.0	17.1
DP-388	Monsanto	76 ghij	32.7 ab	88 abc	40.8 a	82.0	36.8
DP-419	Monsanto	74 ijk	21.3 fgh	70 hij	24.9 cd	72.0	23.1
Erşan-92	KARI	61o	22.7 efg	69 ij	20.1 efgh	65.0	21.4
Flaş	Özbuğday	83 cde	29.3 bc	91 a	25.8 cd	87.0	27.6
Flora	Bayer	78 efghi	26.5 cd	69 ij	26.2 c	73.5	26.4
Julia	Bayer	67 mn	7.9 o	64 jk	6.7 m	65.5	7.3
M-503	NCRI	70 klm	21.3 fgh	84 bcd	25.5 cd	77.0	23.4
Maraş-92	KARI	90 b	15.5 lmn	87 abc	16.8 hijk	88.5	16.2
Nazilli-84-S	NCRI	73 ijkl	19.3 ghijk	89 ab	20.0 efghi	81.0	19.7
Sayar-314	ÇARI	82 cdef	22.7 efg	86 abc	24.4 cd	84.0	23.6
SG-125	Monsanto	85 bcd	25.2 de	79 de	22.2 def	82.0	23.7
ST-373	May Çukonar	47 p	6.4 o	55 l	4.7 m	51.0	5.6
ST-453	May Çukonar	81 defg	12.2 n	77 efg	11.4 l	79.0	11.8
ST-468	May Çukonar	73 ijkl	36.1 a	69 ij	25.5 cd	71.0	30.8
ST-488	May Çukonar	76 ghij	32.8 ab	88 abc	35.3 b	82.0	34.1
Şahin 2000	Özbuğday	76 ghij	20.2 ghijk	71 ghi	18.9 fghi	73.5	19.6
Tex	Özbuğday	63 no	24.9 de	59 kl	23.3 cde	61.0	24.1
Pearson's correlation coefficients							
2008	DI×IVD	r=0.29 P<0.001					
2009	DI×IVD	r=0.53 P<0.0001					

[w] *ÇARI* Çukurova Agricultural Research Institute, *KARI* Kahramanmaraş Agricultural Research Institute, *NARI* Nazilli Cotton Research Institute

[x] DI, disease incidence; IVD, incidence of *Verticillium dahliae*. Verticillium wilt incidence was recorded on 24 September 2008 and 11 October 2009. Percentage was obtained by dividing the number of infected plants by the total number of plants in a row and multiplying by 100

[y] Obtained by plating 1,000 seeds in four replications of 250 seeds each on Sorenson's NP10 semi-selective medium containing chloramphenicol, streptomycin sulfate and chlorotetracycline HCl (50 mg l^{-1}). Identification of *V. dahliae* was made after incubation at 24°C in darkness for 5–7 days

[z] Within columns, means followed by a common letter do not differ significantly at P=0.05 using analysis of variance (GLM) and mean separation (LSD) (SAS Institute, Cary, NC, USA)

general freeze-blotter assay modified from that described by du Toit *et al.* (2005) for detection of *V. dahliae* on spinach seed. To determine the percentage of seed infection by *V. dahliae*, 250 acid-delinted seeds from each genotype per replication were soaked in 1.2% NaOCl for 5 min and rinsed three times for 5 min each time in sterile deionized water. The seeds were dried in a laminar flow hood on sterile paper toweling. One sterile steel blue germination blotter (8.25 cm diam; Anchor Paper Co., St. Paul, MN, USA) was placed within each sterile 9-cm-diam plastic petri plate and moistened with 4 ml of sterile deionized water. Using forceps that were flame-sterilized between seeds, the seeds were transferred onto the blotters (25 seeds per plate) and the plates were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA). The plates were incubated in the dark at 24°C for 24 h to allow the seed to imbibe. The imbibed seed were placed at −20°C for 24 h to kill the embryo and then transferred to an incubator set at 24°C with 12 h:12 hL:D cycle (near-UV and cool-white fluorescent light by day) for 12 days (du Toit *et al.* 2005). The seeds were examined microscopically (×8 to ×100 magnification) approximately 5, 10 and 14 days after plating. *V. dahliae* infections were confirmed by comparing morphological characteristics of colonies with established keys (Hawksworth and Talboys 1970) and recorded. Four replications were plated (for a total of 1,000 seeds per genotype), and the total percentage of seed infection was calculated.

Seed transmission of V. dahliae Two hundred and fifty seeds were selected at random from the infected seed lot of each of the genotypes and germinated in an incubator at 24°C in darkness for 24 h. Two hundred germinating seeds were selected and planted, two seeds per pot, in 100 pots (10 cm diam) containing a steam-pasteurized mixture of soil, peat and sand (1:2:2, v/v). Pots were placed close to each other on greenhouse benches to obtain the thick canopy needed to retain moisture and provide the humid conditions necessary for verticillium wilt development (DeVay and Pullman 1984). Plants were watered daily and fertilized twice per month with 0.2% NH_4NO_3 throughout the growth period. Temperature and relative humidity were monitored by a recording hygrothermograph. The greenhouse temperature ranged from 24° to 34°C by day and from 22° to 26°C at night. These temperatures are within the range necessary for *V. dahliae* to infect when inoculum is present (DeVay and Pullman 1984). Relative humidity averaged 70–85%, a range that favors disease development (Nagtzaam *et al.* 1997; Pérez-Artés *et al.* 2000). Plants showing signs of infection were sampled to determine the cause. Stem and crown segments from these plants were surface-sterilized with 0.5% sodium hypochlorite for 3 min, plated on Sorenson's NP10 semi-selective medium containing chloramphenicol, streptomycin sulfate and chlorotetracycline HCl (50 mg l^{-1}) (Kabir *et al.* 2004; Sorensen *et al.* 1991). Plates were incubated at 25°±1°C in the dark for up to 2 weeks to allow for growth of any microorganisms associated with the infection. Remaining plants were monitored for typical verticillium wilt symptoms. Symptomatic plants were counted to determine the number of infected plants. Disease incidence (DI) was recorded at 3–4-day intervals by counting the number of plants with disease symptoms and expressing this as a percentage of the total number of plants in each treatment; maximum DI was reached when no new infections were detected.

Incidence of V. dahliae in seed harvested from plants grown in the greenhouse At maturity, seeds were harvested from surviving infected plants and air-dried in paper bags on greenhouse benches at 24°C. All seeds were surface-sterilized with 0.5% sodium hypochlorite as described previously and plated on Sorenson's NP10 to determine the percentage of seed infection. Seeds from nonsymptomatic plants were also harvested and plated on Sorenson's NP10 to determine seed infection, even though no above-ground symptoms were observed on these plants.

Statistical analysis Data were analyzed using the general linear model procedure (PROC GLM) of SAS (SAS Institute Inc., Cary, NC, USA). Means were compared using Fisher's protected least significant difference (LSD), where α=0.05. Relationships of the variables among the different trials were evaluated using Pearson correlation analysis (PROC CORR) in SAS.

Results

In 2008 and 2009, the average maximum field DI values recorded for Çukurova-1518, BA-151, Maraş

Table 2 Transmission of verticillium wilt from infected cotton seed in greenhouse tests

Genotype	2008 seed		2009 seed		Average	
	Number of infected plants[y]	Percent transmission	Number of infected plants	Percent transmission	Number of infected plants	Percent transmission
BA-151	15 a[z]	7.5 a	17 a	8.5 a	16	8
Celia	6 b	3 b	7 b	3.5 b	6.5	3.3
Çukurova-1518	18 a	9 a	20 a	10 a	19	9.5
Flaş	19 a	9.5 a	17 a	8.5 a	18	9
Maraş-92	8 b	4 b	10 b	5 b	9	4.5

[y] Total of 200 plants in each test representing four replications of 50 plants each

[z] Within columns, means followed by the same letter do not differ significantly at $P=0.05$ using analysis of variance (GLM) and mean separation (LSD) (SAS Institute, Cary, NC, USA)

92, Flaş and Celia were 95%, 90%, 89%, 87% and 86%, respectively (Table 1). Twenty-nine seed samples belonging to the cotton cultivars most frequently grown in Turkey showed 4.7–40.8% infection with *V. dahliae* when plated on Sorenson's NP10 (Table 1). Coefficients of correlation were low ($r=0.29$, $P<0.001$) for the relationship between disease incidence (%) and incidence of *V. dahliae* (%) in 2008 and moderate ($r=0.53$, $P<0.0001$) in 2009. The highly susceptible cultivar Çukurova-1518 had an average of 32% infested seed, which was significantly less than the highest level of seed infestation detected. The lowest levels of infestation were found in the cultivars Julia and ST-373, which were not significantly different from one another, but were different from the other cultivars tested. ST-373 is considered tolerant to *V. dahliae* (Göre *et al.* 2009). Other fungi commonly associated with seed included species of *Penicillium, Alternaria, Stemphylium, Fusarium* and *Trichoderma* (Fakhrunnisa and Ghaffar 2006; Hyde and Galleymore 1951; Neergaard 1977; Verma and Khan 1965).

Five cultivars with an average DI rate above 85%, were selected for further studies. Seed transmission of verticillium wilt was tested in two greenhouse tests using infected cotton seed harvested from field plots in 2008 and 2009 (Table 2). Because the incidence of verticillium wilt increases over time, five disease incidence readings were taken in each test. Readings began when typical symptoms of wilt first appeared and ended when no new infection was detected. The most characteristic verticillium wilt symptoms included interveinal yellowing before becoming necrotic, stunting, gradual wilting and brownish discoloration of

Table 3 Incidence of *Verticillium dahliae* in cotton seed from greenhouse-grown plants exhibiting symptoms of verticillium wilt and plants without disease symptoms[x]

Genotype	Incidence of *V. dahliae* from harvested seed (%)	
	Symptomatic plants	Nonsymptomatic plants[y]
BA-151	43 b[z]	0 b
Celia	14 c	0 b
Çukurova-1518	55 a	3.5 a
Flaş	64 a	3 a
Maraş-92	61 a	0 b

[x] Obtained by plating all seeds collected from plants of each genotype on Sorenson's NP10

[y] Plants grown in the same pot with infected plants

[z] Within columns, means followed by the same letter do not differ significantly at $P=0.05$ using analysis of variance (GLM) and mean separation (LSD) (SAS Institute, Cary, NC, USA)

Table 4 Transmission efficiency of *Verticillium dahliae* by infected cotton seed in greenhouse test

Genotype	Seed infection (%)[x]	Seed transmission (%)[y]	Transmission efficiency[z]
Test 1 (2008 seed)			
BA-151	24.7 def[w]	7.5 a	30.4 a
Celia	17.6 ijklm	3 b	17 b
Çukurova-1518	27.9 cd	9 a	32.3 a
Flaş	29.3 bc	9.5 a	32.4 a
Maraş-92	15.5 lmn	4 b	25.8 a
Test 2 (2009 seed)			
BA-151	24.4 cd	8.5 a	34.8 a
Celia	20.3 efgh	3.5 b	17.2 b
Çukurova-1518	31.6 b	10 a	31.6 a
Flaş	25.8 cd	8.5 a	32.9 a
Maraş-92	16.8 hijk	5 b	29.8 a

[w] Within columns, means followed by a common letter do not differ significantly at $P=0.05$ using analysis of variance (GLM) and mean separation (LSD) (SAS Institute, Cary, NC, USA)

[x] Obtained by plating 1,000 seeds in four replications of 250 seeds each

[y] Total of 200 plants in tests representing four replications of 50 plants each

[z] Subsamples of seed were plated on Sorenson's NP10 to obtain values for seed infection, while a second subsample from the same seed lot was planted. Transmission efficiency was calculated by dividing seed transmission by seed infection and multiplying the result by 100

vascular tissues, observed on plants from the 2008 seed lot in early August, about 120 days after planting. Symptoms were observed in plants from the 2009 seed lot in mid August, about 130 days after planting. These symptoms included wilting and subsequent plant death. Microsclerotia formation was observed in xylem vessels of cotton stems shortly after signs of the disease were observed on the leaves.

Verticillium dahliae was isolated from seeds of infected plants of all genotypes that showed some degree of seed transmission (Table 3). The pathogen was also isolated from some seed of nonsymptomatic plants among genotypes that showed the capability for seed transmission. The percentage of incidence of *V. dahliae* was higher in seed from symptomatic plants than in those from nonsymptomatic plants (Table 3). Pathogen recovery was significantly lower in seeds of the genotype Celia than the others. As before, *Penicillium, Alternaria, Stemphylium, Fusarium* and *Trichoderma* were present in plated seeds of symptomatic and nonsymptomatic plants.

The transmission efficiency of verticillium wilt was calculated for each genotype by dividing the percentage of seed infection (determined by plating a subsample of seed on Sorenson's NP10) by seed transmission (determined by plating a second subsample of the same seed lot) and multiplying the result by 100. BA-151, Çukurova 1518, Flaş and Maraş 92 showed high values for transmission efficiency in both tests (Table 4). Despite a high percentage of seed infection in Celia, this genotype had a transmission efficiency of 17%; low seed transmission of *V. dahliae* was obtained in the greenhouse in both tests.

Discussion

The annotated list of seedborne diseases published in 1990 (Richardson 1990) records almost 1,500 seedborne microorganisms on approximately 600 genera of agricultural, horticultural and tree crops. From the plant quarantine standpoint, these figures emphasize the magnitude of the problems involved in controlling the movement of seedborne pathogens into areas where they have not previously been recorded.

Diseased seeds can sometimes be detected by visual examination of dry seed, but this way of assessing seedborne inoculum is rarely sensitive enough to be of practical value (McGee 1981). Most

tests involve plating seeds on culture media. Serological tests for detection of seedborne pathogens also have been developed (Carroll *et al.* 1979). In addition, laboratory testing procedures have been developed for many seedborne pathogens (Agarwal and Sinclair 1997). However, the values obtained in laboratory tests cannot always be related to the risk of disease development in the field. A test that provides the highest pathogen count on media may not be the most useful test for predicting field disease. In our study, for example, low seed transmission of *V. dahliae* was recorded on Celia, even though this genotype showed high seed infection. Thus, we cannot deduce potential for seed transmission based on media counts of seed infection alone. The transmission efficiency of each genotype gives an idea of what to expect when evaluating contaminated seed for seed transmission. Our results showed that different genotypes exhibited different transmission efficiencies, which implies that seed transmission of *V. dahliae* may be genotype-dependent. Unless epidemiological studies are conducted to relate the results of laboratory seed infection tests to the actual risk of subsequent field disease, laboratory tests will continue to be of little practical value.

We do not know for sure how fields newly brought into cultivation become infested with *V. dahliae*, but it is possible that long-distance spread of the pathogen could be a result of infected seed. Our study shows that spread by infected seed is possible under greenhouse conditions that favor disease development. However, the discovery of *V. dahliae* in a field need not be connected to recent seed introduction. *Verticillium* spp. have a wide host range (Bhat and Subbarao 1999), and *V. dahliae* may have been present in the field in low numbers until changes in the environment or farming practices enabled its development (Pegg 1974).

It is possible that fields in the Aegean, Mediterranean and southeastern Anatolia regions—where cotton was grown for years with no evidence of verticillium wilt—now sustain severe losses because contaminated seed was brought in from severely infected fields. Because of this, it is important to consider carefully the source of seed before planting in disease-free fields or in new cotton fields. It may also be important to consider whether crops that are rotated with cotton are potential hosts of *V. dahliae* able to introduce the pathogen into noninfested fields.

Acknowledgments This project was supported by the General Directorate of Agricultural Research of Turkey. Our thanks are expressed to Mr. Ercan Gül for technical assistance.

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