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THE RELATIONSHIP OF VERTICILLIUM WILT AND SEED SURFACE MICROFLORA WITH GOSSYPOL LEVEL IN COTTON (*GOSSYPIUM* SPP.)

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ABSTRACT

The aim of study was determined to relationship between on the seed surface microflora and reaction to Verticillium wilt of cotton cultivars with different gossypol levels. Thirteen cotton cultivars were examined gossypol level and the seed surface microflora of the cotton *in vitro*. Then, these cultivars were observed susceptibility to non-defoliating (Vd11 isolate) and defoliating (PYDV6 isolate) pathotypes of *Verticillium dahliae* Kleb. *in vivo*. Cultivars were significant at ($P \leq 0.05$) probability level for disease intensity values *in vivo* and gossypol values *in vitro*. While the highest gossypol value was determined in "Gloria" cultivar (*Gossypium hirsutum* L.) with a 1.669 µg $mL^{-1}$and resistant control "Giza 45" (*Gossypium barbadense* L.) with a 1.343 µg mL^{-1}, the lowest gossypol value was found in "Gossypolsüz Nazilli" (0.196 µg mL^{-1}) and susceptible control "Çukurova 1518" (0.484 µg mL^{-1}) of *G. hirsutum* L. While the lowest of disease intensity value was observed Vd11 isolate and PYDV6 isolate in the highest gossypol, resistant control "Giza 45" cultivar (0.30-1.11). The highest disease intensity values were found in the lowest gossypol, "Gossypolsüz Nazilli" cultivar (2.24-2.82) and susceptible control "Çukurova 1518" cultivar (2.00-2.63), respectively. Fungus species were isolated in high and low gossypol of cotton seeds at the same rate.

KEYWORDS:
Gossypol, *Verticillium dahliae* Kleb., disease intensity, cotton.

INTRODUCTION

Cotton, outside the textile industry is the raw material of approximately 50 industries such as oil, gunpowder and film material. Cotton oil is used in many areas such as soaps and detergents and animal feed. Cotton oil, as an alternative to petrol, is increasingly being used as raw material in bio-diesel production too [1]. About 34 million ha of cotton growing and obtained from approximately 24.8 million tons of lint yield from this area in the world. India, China, USA, Pakistan, Brazil, Uzbekistan and Turkey is one of the leading countries in cotton production [1, 2]. In Turkey, about 458.000 ha of upland cotton was grown and 738.000 tons lint yield was produced in the four main regions Southeastern Anatolia, Aegean, Çukurova and Antalya. Turkey's share in world cotton production is approximately 2.5 % and is ranked seventh [3].

Gossypol is a phenolic compound triterpenoid aldehyde or polyphenolic binaphthyl aldehyde in both plant and seeds in all types of cotton grown. This compound was synthesized by the epidermal cells in the roots, it is communicated to the interior decoration of transmission and here it is localized. Firstly, Gossypol defined in 1886 by Longmore and purified by Marchlewski in 1889 [4]. No gossypol plants were obtained by Michael for the first time in 1954. Smith [5] reported the lowest rate of gossypol on root and the highest rate of gossypol on seed in plant. Sotelo et al. [6] observed the amount of gossypol in the leave with 0.297 mg g^{-1}, in the seed with 0.847 mg g^{-1} of *G. hirsutum* L. Fidan et al. [7] concluded that gossypol concentration of the seed varies from 0.0 % to 9.0 %, but most commercial cotton varieties usually contain the compound from 0.6 % to 2.0 %. Gossypol not only to human and animals, but also can be active against pests, fungi and microorganisms. Many researchers reported that gossypol have antimicrobial, anticancer and antioxidant properties [8, 9, 10]. Also, the pigment glands of cotton plants includes derivatives with gossypol such as desoxyhemigossypol, hemigossypol, hemigossypolone and heliocide. These compounds are important in plant protection against pests and diseases [11].

V. dahliae Kleb. is one of the factors that negatively affect yield and quality in cotton growing, known worldwide as the most devastating and destructive, causing wilt in 160 families and 40 different plant species [12, 13]. Pathogen, invaded xylem, cause xylem occlusion, so major damage occurs on plant yield and quality [14, 15, 16, 17].

The losses of seed cotton yield due to Verticillium wilt were reported about 75 % in California, 8- 10 % in Russia and 4 % in Syria [18]. Verticillium wilt was responsible for significant yield losses (approximately 1.5 million bales) in the world cotton belt [19]. One of the most effective methods is used to resistant varieties without an economic chemical control in the control against the Verticillium wilt. Several authors reported that varieties lose resistance over time and development of resistant varieties studies should be made permanent [20, 21, 22]. Also, in Turkey, cotton is grown in different climatic conditions, causing damage to drought and heat, and this is effective in the selection of cotton varieties [23].

Today, the number of studies of susceptibility to Verticillium wilt containing of gossypol levels in cotton cultivars is very limited. Desoxyhemigossypol and hemigossypol synthesized in plants against pathogenic fungi such as *V. dahliae* and *Fusarium oxysporum* f.sp. *vasinfectum*, these pathogens were classified as phytoalexin, because they are also toxic [24]. Puckhaber et al. [25] carried out to determine the effect of gossypol on *Rhizoctonia solani* development of positive and negative in the studies, reported gossypol may equally positive and negative effect, whereas seen gossypol inhibition less than desoxyhemigossypol and hemigossypol, hemigossypol and desoxyhemigossypol has the most important fungicide. A positive gossypol was found better bactericide by negative gossypol [26].

The aim of study was determined to relationship between on the seed surface microflora and susceptibility to non-defoliating (ND) and defoliating (D) pathotypes of *V. dahliae* Kleb. of cotton cultivars with different gossypol levels.

MATERIALS AND METHODS

Materials. Plant materials. In the study, a total of thirteen cotton cultivars were used as control of three different cultivars (Carmen/tolerant-Bayer Crop Science AG, Leverkusen, Germany; Çukurova 1518/susceptible-Eastern Mediterranean Agricultural Research Institute, Adana, Turkey and Giza 45/resistant-Cotton Research Institute, Egypt) of different cotton species as material (Table 1).

Test microorganism. Isolated from cotton and a high virulence pathotypes, Vd11 isolate (ND pathotype, Nazilli Cotton Research Institute) and PYDV6 isolate (D pathotype, Adnan Menderes University, Faculty of Agriculture, Department of Plant Protection) used for an artificial inoculum [27].

Detection of gossypol level on cotton seeds. Cotton seeds of each cultivar were weighed 1 g in experiment, these were homogenized in 10 mL of 96 % ethanol. Extract was removed and the residue passed through two layers of cheesecloth. After filtration, the extracts were extracted three times with 1 volume diethyl ether (1:1, v:v). The ethanol was removed by rotary evaporator [28]. Extraction procedures were performed in three replications for each cotton varieties. Liquids containing various concentrations gossypol (0-2 ppm) were used for determining quantities of gossypol as standard. The resulting liquid UV visible spectrophotometer (T-80 Plus UV-VIS Spectrophotometer, PG Ins Ltd LA) was observed at 440 nm and quantitated by gossypol [29].

Pot trials. Conidial suspension technique was used to test the susceptible of cotton cultivars with different percentage levels of gossypol against *V. dahliae* Kleb. in pot trials. Experiment was established as randomised plot design with five replications in a growth room (at 24±1 °C; 50-70 % relative humidity; a 12 h light/12 h dark). A 33 % soil, 33 % sand and 33 % peat containing mixture was sterilized in autoclave at 121°C for one hour and was filled into 5 cm diameter plastic pots. Of the 4 seeds sown each plastic pots, only one left at cotyledonary stage and others were removed. *V. dahliae* cultures having high virulence isolates (Vd11 and PYDV6) were developed on Potato Dextrose media broth for inoculation. After two weeks, spore suspension in the flask were adjusted as 4 x 10^6 conidia mL^{-1} with a hemacytometer and 5 mL of adjusted suspension released to the bottom of each plastic pots and plants at the six-true-leaf stage were placed in the pots. Three-five weeks after inoculation, disease severity was assessed for each plant on a 0-to-4 rating scale according to the percentage of foliage affected by acropetal chlorosis, necrosis, wilt, and/or defoliation (0=healthy plant; 1=1–33 %; 2=34–66 %; 3=67–97 %; 4=dead plant) [30]. Disease Severity Index (DSI) was calculated using the formula and obtained data were subjected to Arcsin for transformation [31].

DSI=[(ax0)+(bx1)+(cx2)+(dx3)+(ex4)]/M

DSI: Disease severity index; a, b, c, d, e: The plant number with degree 0, 1, 2, 3, 4 respectively, M= Total plant number.

Detection of microflora on cotton seed surface. Seeds of each cultivar in order to determine microflora of cotton seed heated for 5 minutes in 5 % NaOH and a waiting period on sterile blotting excess moisture is received after washing twice with sterile distilled water. Seed surfaces after drying were cultured on Potato Dextrose Agar (PDA) media and the seeds were incubated at 25 ± 1°C for 10 days. 100 seeds were used for each genotype in this method. Growing colonies at the end of incubation period were counted to genus level and isolating ratios are calculated in the experiment. At the species level identification of isolated fungi were

carried out using macroscopic and microscopic methods [32, 33, 34, 35].

Statistical analysis. All data obtained in experiments were analysed statistically by using JMP statistical software program (5.0.1, SAS Institute, Cary, NC) for analysis of variance and means were compared using Fisher's protected least significance difference (LSD) test at 5 % probability level [36].

RESULTS AND DISCUSSION

Detection of gossypol level on cotton seeds. The levels of gossypol (µg mL^{-1}) in the seeds of cotton cultivars in the experiment were given in Table 2. The amounts of gossypol have varied according to the species and cultivars of the same species in cotton seeds. Cultivars were significantly ($P \leq 0.05$) different level for gossypol results. The lowest amount of gossypol was determined in "Gossypolsüz Nazilli" cv (*G. hirsutum*) with a 0.196 µg mL^{-1} and susceptible control "Çukurova 1518" cv (*G. hirsutum*) with a 0.484 µg mL^{-1} in the cotton seeds. While the highest amount of gossypol was observed in "Gloria" cv (*G. hirsutum*) with a 1.669 µg mL^{-1} and resistance control "Giza 45" cv (*G. barbadence*) with a 1.343 µg mL^{-1}, respectively and followed by "St-373" cv (1.090 µg mL^{-1}) and "GSN 12" cv (0.911 µg mL^{-1}). Other candidate cultivars had gossypol levels between 0.599 µg mL^{-1} and 0.850 µg mL^{-1} (Table 2). In a similar study, gossypol level was observed between 0.81 % - 1.04 % in *G. barbadense* L. and 0.64 % - 1.09 % in *G. hirsutum* L. in the seeds studies [37]. Gossypol level with 0.0 % type of *G. sturtian* Will. of Australian origin was determined more than 9.0 % type of *G. davidsonii* Kell. [38]. Gossypol level was found a 0.384 mg g^{-1} in "Mexico 72-69" (*G. barbadense* L.), a 0.218 mg g^{-1} in "SP-21" (*G. hirsutum* L.) [9]. The amount of gossypol in cotton plants and seeds which can vary across varieties [39]. The amount of gossypol was determined a 0.387 mg g^{-1} and a 6.780 mg g^{-1} in the leaves and seeds of

TABLE 1
Species and origins of cotton cultivars in the experiment.

Cultivar (cv)	Species	Origin
Gloria	*G. hirsutum*	Australian
Carmen (tolerant-control)	*G. hirsutum*	Australian
Nazilli DT-15	*G. hirsutum*	Nazilli-Turkey
Çukurova 1518 (susceptible-control)	*G. hirsutum*	Adana- Turkey
Flash	*G. hirsutum*	Hatay- Turkey
Maydos Yerlisi	*G. herbaceum*	Nazilli- Turkey
Giza 45 (resistant-control)	*G. barbadence*	Egypt
Gossypolsüz Nazilli	*G. hirsutum*	Nazilli- Turkey
NP Özbek 100	*G. hirsutum*	Nazilli- Turkey
BA 308	*G. hirsutum*	Hatay- Turkey
St-373	*G. hirsutum*	USA
GSN 12	*G. hirsutum*	Nazilli- Turkey
Cloudia	*G. hirsutum*	Australian

TABLE 2
Gossypol level of cotton seeds in the experiment (µg mL^{-1}).

Cultivar (cv)	Gossypol level (µg mL^{-1})[a]
Gloria	1.669 a
Carmen (tolerant-control)	0.599 ef
Nazilli DT-15	0.850 bcd
Çukurova 1518 (susceptible-control)	0.484 f
Flash	0.764 cde
Maydos Yerlisi	0.701 cdef
Giza 45 (resistant-control)	1.343 a
Gossypolsüz Nazilli	0.196 g
NP Özbek 100	0.722 cdef
BA 308	0.769 cde
St-373	1.090 b
GSN 12	0.911 bc
Cloudia	0.630 def

[a] Mean values with the same letter within a column are not significantly different at the 0.05 probability level by LSD.

TABLE 3
Disease intensity values of cotton cv inoculated with two pathotypes of *V. dahliae* isolates in pot trials.

Cultivar (cv)	Disease intensity values in pot trials	
	Vd11 isolate[a]	PYDV6 isolate[a]
Gloria	0.72 f	1.58 ef
Carmen (tolerant-control)	0.60 g	1.51 f
Nazilli DT-15	0.72 f	1.60 ef
Çukurova 1518 (susceptible-control)	2.00 b	2.63 b
Flash	0.70 f	1.57 ef
Maydos Yerlisi	0.60 g	1.51 f
Giza 45 (resistant-control)	0.30 h	1.11 g
Gossypolsüz Nazilli	2.24 a	2.82 a
NP Özbek 100	1.50 c	2.25 c
BA 308	0.89 d	1.79 d
St-373	0.70 f	1.56 ef
GSN 12	0.85 de	1.77 d
Cloudia	0.77 ef	1.65 de

[a] Mean values with the same letter within a column are not significantly different at the 0.05 probability level by LSD.

TABLE 4
Rates of fungi growing on the seed surface in PDA media (%).

Cultivar (cv)	Growing rates of fungi in seed (%)							
	Fs	Rh	Al	As	Tr	Rz	Ng	Others[a]
Gloria	18	5	39	24	2	1	5	10
Carmen	18	6	28	26	7	2	-	13
Nazilli DT-15	20	4	31	27	3	1	6	8
Çukurova 1518	19	8	33	26	2	2	-	10
Flash	18	3	33	33	3	1	-	9
Maydos Yerlisi	25	6	37	28	4	3	7	10
Giza 45	28	2	35	21	5	1	-	8
Gossypolsüz Nazilli	30	12	30	15	5	1	3	4
NPÖzbek 100	12	4	35	24	5	1	4	15
BA 308	35	6	20	29	1	1	6	2
St-373	28	7	31	25	2	4	-	3
GSN 12	25	4	35	26	2	3	-	5
Cloudia	22	3	33	30	3	3	3	3

Fs: Fusarium, Rh: Rhizoctonia, Al: Alternaria, As: Aspergillus, Tr: Trichotechium, Rz: Rhizopus, Ng; Nigrospora.
[a]Growing bacteria, yeast colonies in seeds on PDA media.

"Acala 1517-70"; a 6.780 mg g^{-1} and a 10.980 mg g^{-1} in the leaves and seeds of "OR-19", respectively [40].

Pot trials. Disease intensity index values of cotton cultivars in pot trials were given in Table 3. Differences between cultivars were significant at ($P \leq 0.05$) probability level for disease severity value in a pot experiment conducted in a growth room. In the pot experiments, disease resistant and high amount of gossypol, resistant control "Giza 45" cv with a 0.30 index value showed the lowest disease severity and followed by "Carmen" cv (0.60) and "Maydos Yerlisi" cv (0.60) for Vd11 isolate according to the disease severity index. The highest intensity of disease was observed in low amount of gossypol, "Gossypolsüz Nazilli" cv (2.24) and susceptible control "Çukurova 1518" cv (2.00). Other candidate cultivars had index values between 0.70 and 1.50. Resistant control "Giza 45" cv (1.11) was in the first place and followed by tolerant control "Carmen" cv (1.51) and "Maydos Yerlisi" cv (1.51). Again the highest intensity of disease was determined in "Gossypolsüz Nazilli" (2.82) and susceptible control "Çukurova 1518" cv (2.63) for PYDV6 isolate. Zaki et al. [41] reported the antifungal compounds determining in inoculated plants with *Verticillium albo-atrum*, these compounds could not detect in uninoculated plants. Also, vergosin and hemigossypol compounds were more effective than the antifungal agents and gossypol against *V. albo-atrum*. According to Mace and Stipanovic [42] found desoxyhemigossypol (6.1 µg ml^{-1} dose) in cotton roots and stems infected with *V. dahliae* reduced growth of the pathogen around 75 %. When used to different concentrations of some herbicides in cotton areas applied to cotton seed, haloxyfob and linuron have prevented to the mycelial growth of *F. oxysporum* f. sp. *vasinfectum* in solid and liquid culture. The amount of gossypol

increased compared to control in disease plants, which prevented to the mycelial growth of the pathogen *in vitro* [43]. The peptide components have isolated resistant to fungal pathogens and bollworm seeds of the eight cotton varieties and when capable of inhibition *V. dahliae* conidia, α-amylase and other peptides viewed, between fungicidal activity of peptides and resistance of cotton varieties determined a correlation [44]. Ten cotton varieties were inoculated with D and ND pathotypes (10^6 conidia mL^{-1}) in the growth chamber, As a result of the study, the lowest disease severity value was determined in the "Maydos Yerlisi" cv [45].

Detection of microflora on cotton seed surface. Rates of fungi growing on the seeds cultured on PDA medium were given in Table 4. *Alternaria* spp., *Fusarium* spp. and *Aspergillus* spp. isolated intensively both low levels of gossypol and high levels of gossypol on seed, respectively. *Alternaria* spp. were found the highest in "Gloria" cv (39 %), the lowest in "BA 308" cv (20 %). *Fusarium* spp. were determined the highest in "BA 308" cv (35 %) and "Gossypolsüz Nazilli" cv (30 %). In similarly, *Aspergillus* spp. were isolated between 15 % - 33 % in low and high gossypol of cultivars. Macroscopic and microscopic studies of carried out on cotton seed; Alternaria colonies were identified as largely *A. alternata*; *Fusarium* spp. were identified as *F. oxysporum*, *F. semitectum* and *F. solani*; *Aspergillus* spp. were identified as *Aspergillus niger*, *A. flavus* and *A. ochraceus*. Isolating rate of *Rhizoctonia* spp. was found between 2 - 12 % in low and high gossypol of cultivars (Table 4). Our results showed parallels with the other studies. Moore and Rollins [46] reported that outside capillary roots and seed coat of gossypol in the cotton plant, as the internal structure of seeds, leaves, stems, roots, branches, boll shell, stigma and the sytle in plant and flower parts. Klich [47] was isolated different filamentous fungi in delinted surface sterilized cotton seeds and there were no differences in fungal flora among cultivars. Of the seventheen taxa isolated, *Alternaria* spp. *Colletotrichum gossypii*, *Fusarium equiseti*, *F. pallidoroseum* (*F. semitectum*) were present more than 10 %. *F. semitectum* have isolated a sulfuric acid delint in the cotton seed by blotter method, but haven't determined a relationship with disease symptoms after the seed output in the autoclaved soil [48]. Arabsalmani [49] isolated that fungal species of different genus from seed coat and embryo parts on PDA medium after surface disinfection of seeds in the study. While *A. alternata* and *Fusarium* spp. were isolated from embryo portion of "Sahel" cv by 32.1 % and 33.3 % ratio, *A. macrospora* and *Fusarium* spp. were isolated from the seed surface by 25 % and 21.4 % ratio, respectively. The absence of a relationship between gossypol level and fungus developing on the seed surface may be attributed to environmental and climatic conditions. Similar results were reported by Baba [50].

CONCLUSION

In this study, the amount of gossypol in the cotton seed was determined different to species, the cultivars of the same species and origins. While the highest amount of gossypol determining both *G. barbadence* L. (tolerant control Giza 45 cv) and *G. hirsutum* L. (Gloria cv), the lowest amount of gossypol has been determined in "Gossypolsüz Nazilli" cv and susceptible control "Çukurova 1518" cv (*G. hirsutum* L.). In this context, gossypol content of the seed may vary according to species, variety, environmental conditions, plant growth and development stages. While the lowest disease severity against both pathotype of *V. dahliae* Kleb. was determined on the highest amount of gossypol in resistant control "Giza 45" cv, the highest intensity of disease was found on the lowest amount of gossypol in "Gossypolsüz Nazilli" cv. The disease intensity index values were obtained near the *G. barbadance* L. such as "Gloria", "Carmen" and "Claudia" cultivars with origin of Australian in upland cotton. Plant breeders will increase the chances of success in the resistance breeding studies against the Verticillium wilt, especially the use of cultivars with high gossypol in the cotton plant parts without an economic chemical control in the control against Verticillium wilt. Meanwhile, fungi were isolated on the seeds of low and high gossypol cultivar at the same level. Therefore, there was concluded any relationship between level of gossypol on the seed and species of fungi growing on the seed surface.

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