Evaluation of Verticillium wilt resistance in commercial cultivars and advanced breeding lines of cotton

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Abstract Verticillium wilt (VW), caused by *Verticillium dahliae* Kleb, is one of the most destructive diseases in cotton (*Gossypium* spp.). The most efficient and cost-effective method of controlling the disease is the use of resistant cotton cultivars. Most commercial cultivars and elite breeding lines are developed under non-VW conditions and their responses to the disease are currently unknown. This study was conducted to evaluate current commercial cotton cultivars and advanced breeding lines for VW resistance. In 2011–2013, a total of 84 cultivars from major US seed companies, 52 advanced breeding lines from the US public breeding programs, and 87 introgression lines

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from a cross between Acala 1517-99 × Pima PHY 76 from the New Mexico Cotton Breeding Program, were evaluated for VW resistance in the greenhouse. Cotton cultivars and breeding lines were evaluated in ten separate replicated tests by inoculation with a defoliating-type isolate of *V. dahliae*. While leaf severity rating and percentages of infected plants, infected leaves and defoliated leaves were found to be significantly and positively correlated with one another, leaf severity rating and percentage of infected leaves were best choices because of their relatively low coefficients of variation and higher resolutions to differentiate resistant genotypes from susceptible ones. The heritabilities for the VW resistance traits ranged from 0.58 to 0.80 with an average of 0.67, indicating that variation in VW resistance is predominantly due to genetic factors. Of the 223 commercial cultivars and advanced lines, six Upland cultivars (FM 9160B2F, FM 9170 B2F, NG 4010 B2RF, Nitro 44 B2RF, DP 1219 B2RF, and ST 4288 B2F), five advanced lines (Ark 0403-3, MD 10-5, MD 25ne, NC11AZ01, and PD 0504), two introgression lines from Upland × Pima (NM11Q1157 and 08N1618), and four Pima cultivars (COBALT, DP 357, PHY 800, and PHY 830) had higher levels of resistance to VW. The resistance shown by most of these cultivars in the greenhouse was consistent with their performance in previous field tests. Based on the initial VW resistance, 19 highly or moderately resistant genotypes were chosen for re-evaluation and 30 genotypes were also assessed more than once for VW resistance in different tests, most of

which had concordant performance. These cultivars and advanced lines should be useful resources to improve VW resistance in cotton breeding.

Keywords Cotton · Germplasm · Verticillium wilt · Resistance

Introduction

Cotton (*Gossypium* spp.) is the most widely cultivated fiber crop in the world. Over 80 countries are growing cotton with Upland cotton (*G. hirsutum* L.) as the predominant type. Verticillium wilt (VW), a fungal disease caused by *Verticillium dahliae* Kleb., has become one of the main constraints in cotton production. The disease caused 0.5–3.5 % yield loss nationwide in the US (Blasingame and Patel 2005) and as high as 7 % in other regions (Karademir et al. 2012). VW also significantly reduced fiber quality including fiber length and micronaire (Zhang et al. 2012). Many methods, such as crop rotation, chemical fumigation, and soil amendments, have been applied to control this disease. But none are effective (Lüders et al. 2008), due to a wide host range of the pathogen, long-term persistence of its resting structure known as microsclerotia, and the inability of fungicides to affect the pathogen once the plants are infected.

The most efficient and cost-effective method of controlling the disease is the use of resistant cotton cultivars. Identifying sources of disease resistance has been successful to improve VW resistance in other crop species (Colella et al. 2008; Gossen and Jefferson 2004; Miranda et al. 2010). For example, a resistance gene *Ve*, which confers resistance to some isolates of *V. dahliae*, has been cloned in tomato, *Solanum lycopersicum* L. (Diwan et al. 1999). Of the four cultivated cotton species (*G. hirsutum*, *G. barbadense* L., *G. arboretum* L., and *G. herbaceum* L.), only *G. barbadense*, known as Pima cotton in the US, has a higher level of resistance against VW (Wilhelm et al. 1974). But it is planted in very limited areas in California, as well as in the arid regions of Southwest Texas, New Mexico, and Arizona (USDA-ERS 2013). Resistant traits from Pima cotton have not been successfully transferred into commercial Upland cotton (Zhang et al. 2012). Since the 1930s, Acala cultivars have been released in California and New Mexico for cotton production (Oakley 1998; Zhang

et al. 2005). Due to high yield, good fiber quality and resistance to VW, these Acala cultivars were used to control VW. However, Zhang et al. (2012) reported that only some Acala cotton conferred resistance to VW.

Upland cotton is widely planted throughout the US Cotton Belt, accounting for about 97 % of the annual cotton crop (USDA-ERS 2013). However, highly resistant cultivars to VW are lacking and no source of heritable immunity has been found in Upland cotton (Wilhelm et al. 1974). Great efforts have been made to improve the resistance in Upland cotton against VW worldwide (Zhang et al. 2013a, b). Zhang et al. (2012) reported that the VW resistance in Pima was successfully transferred to Upland cotton lines by advanced backcrossing and selfing, and Fang et al. (2013a, b) recently have mapped two quantitative trait loci (QTL) for VW resistance in an interspecific backcross inbred line population and 21 QTL associated with VW resistance in an introgressed inbred line population. However, whether VW resistance can be transferred from Pima to Upland cotton through pedigree selection is currently unknown. Information on levels of resistance against VW is lacking for most newly released commercial cultivars, most of which were developed under non-VW conditions. The same is true for advanced breeding lines developed from the public cotton breeding programs in the US. VW disease resistance genes may have been randomly fixed during the selection process. The objective of this study was to evaluate VW resistance in current commercial cotton cultivars, advanced breeding lines, and introgression lines from a cross between Pima PHY 76 and Acala 1517-99 under greenhouse conditions.

Materials and methods

Plant materials

A total of ten replicated experiments were conducted in the greenhouse in 2011 (three tests), 2012 (four tests) and 2013 (three tests).

2011 experiments

Seventy-six entries were divided into three tests (11Test 1—Trial HQ, 11Test 2—Trial RB, and 11Test 3—Trial B) to evaluate VW resistance in the

greenhouse at Fabian Garcia Science Center, New Mexico State University (NMSU), Las Cruces, NM. 11Test 1—Trial HQ included 32 commercial Upland cotton cultivars, provided by seed companies for the National Cotton Variety Test (NCVT) and High Quality Test (HQ). 11Test 2—Trial RB had 34 advanced Upland breeding lines from the Regional Cotton Breeders' Testing Network (RBTN) provided by the US public cotton breeding programs. 11Test 3—Trial B contained ten Pima cotton (*G. barbadense* L.) cultivars and breeding lines. All the genotypes from the greenhouse test were evaluated in the field for yield and fiber quality in 2010. The greenhouse tests were randomized complete block designs (RCBD) with 4 replicates. The seeds were planted in a 4-in. plastic pot with 10 seeds/pot (5 hills/pot, 2 seeds/hill) in the greenhouse on Sept. 15, 2011. The pots were filled with potting soil (Scott 450, Scotts Co., Marysville, OH, USA) mixed with slow release Osmocote® fertilizer (Marysville, OH, USA). After emergence, seedlings were thinned to 5–6 plants/pot.

The pathogen, *V. dahliae*, which was isolated from an infected cotton plant at Leyendecker Plant Science Center, Las Cruces, NM, was cultured in Czapek—Dox broth at 25 °C at 140 rpm on a rotary shaker for 20 days. The conidial suspension was passed through a double-layer of cheesecloth to separate spores (conidia) from mycelia. Concentration of conidia was adjusted by using a hemacytometer. When seedlings were at the 2nd/3rd true leaf stage, root inoculation was made by pouring 100 mL/pot of conidial suspension to the soil surface. Double inoculations were carried on Oct. 26 (3.45×10^6 conidia/mL) and Nov. 10, 2011 (1.71×10^6 conidia/mL) to ensure that every plant was infected. Assessment of the leaf severity of wilt symptoms was done 41 days after the first inoculation (DAI) on Dec. 6, 2011. As with other greenhouse tests in 2012 and 2013, no un-inoculated control plants were included in these tests, and no leaf symptoms from un-inoculated plants were ever noted in our other greenhouse tests (e.g. Tiwari et al. 2013; Zhang et al. 2013a, b).

2012 experiments

Eighty-seven advanced breeding lines from a cross between Acala 1517-99 × Pima PHY 76 were divided into three different tests (12Test 1—Trial G, 12Test 2—Trial K, and 12Test 3—Trial H), and each test

included 29 lines, two parents, and a common check Acala 1517-08. The seeds used in the greenhouse study were arranged in a RCBD with 3 replications. Seed was planted on Jan. 28, 2012, using the same method as in 2011. Seedlings were thinned to 7–8 plants per pot after seedling emergence. Inoculation was made on March 16 (3.5×10^6 conidia/mL) for these tests and VW resistance was screened on April 20 (i.e. 35 DAI).

Based on the screening results from 2011, 19 high resistant or moderately resistant genotypes were chosen to further screen for VW resistance in the fourth test (12Test 4). Seeds were sown in a RCBD with four replicates on May 12, 2012. The seedlings were inoculated with *V. dahliae* on June 3, 2012 (2.5×10^6 conidia/mL) and June 9, 2012 (3.2×10^6 conidia/mL). The leaf severity was evaluated on July 5, 2012 (32 DAI).

2013 experiments

Ninety-six commercial cultivars and advanced breeding lines were divided into three tests (13Test 1—Trial NV, 13Test 2—Trial RB, and 13Test 3—Trial HQ) for VW disease evaluation. Each of the three tests was arranged in a RCBD with four replications. For 13Test1—Trial NV, cultivars provided by seed companies were planted on March 2, 2013, and plants were inoculated on March 25, 2013 (7.0×10^6 conidia/mL) and on April 3, 2013 (4.8×10^6 conidia/mL). The response of seedlings to infection by the pathogen was evaluated on April 29, 2013 (35 DAI). For 13Test 2—Trial HQ and 13Test 3—Trial RB, seed of cultivars was provided by seed companies and the US public cotton breeders, respectively, and was planted on March 15, 2013. Double inoculations were made for each test on April 9, 2013 (4×10^6 conidia/mL) and April 16, 2013 (6×10^6 conidia/mL). The leaf severity was assessed on May 16 and 17, 2013 (38 DAI). Using vascular discoloration in the stem, the stem severity in the above three tests was evaluated again on May 31, 2013 (67 DAI for Test 1 and 52 DAI for other two).

Evaluation of Verticillium wilt resistance

After 30–40 days following inoculation, the reactions of seedlings to VW were evaluated using a 0–5 rating scale described by Zhang et al. (2012) as follows:

0 No symptom

1 <25 % chlorotic/necrotic leaves, no leaf or cotyledon abscission

2 25–50 % chlorotic/necrotic leaves, less than 2 leaves including cotyledons abscised

3 50–75 % chlorotic/necrotic leaves, 2–3 leaves including cotyledons abscised

4 >75 % chlorotic/necrotic leaves, more than 3 leaves including cotyledons abscised

5 Complete defoliation or plant death

For the three tests in 2013, the vascular discoloration of stems was also used to assess the stem severity following the protocol of Yang et al. (2008) as the follows:

0 no discoloration in the vascular tissue

1 25 % vascular area turns to brown

2 26–50 % vascular area turns to brown

3 51–75 % vascular area turns to brown

4 ≥76 % vascular area turns to brown

For the tests in 2012, plants were only evaluated for VW leaf severity rating. For the tests in 2011 and 2013, data were collected on an individual plant basis for the following traits: the total number of leaves, the number of infected plants, infected leaves and defoliated leaves, and disease severity rating (leaf rating and/or stem rating). For each replicate, percentages of infected leaves, defoliated leaves and infected plants for each genotype were then calculated; and the disease rating for each genotype was calculated as the sum of disease rating scores for all plants divided by the number of plants.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The least significant difference at the 0.1 significance level was used to compare means between cultivars or genotypes. The means in each test were also used to perform a simple correlation analysis between VW resistance traits.

Based on ANOVA, the broad-sense heritabilities were estimated using the following formula:

$$\text{Heritability} = \text{MS(genotype)}/[\text{MS(genotype)} + \text{MS(error)}]$$

Here, MS (genotype) is the mean square of genotype, and MS (error) is the mean square of experimental error. Heritability is calculated by MS (genotype) divided by the sum of MS (genotype) and MS (error).

Results and analysis

Evaluation of VW resistance in commercial Upland cotton

In 11Test 1—Trial HQ, genotypic differences in percentage of defoliated leaves and leaf severity rating at 41 DAI were significant or near significant at $p = 0.10$ level based on ANOVA, while percentages of infected plants and leaves was not significantly different among the 32 cultivars (Supplementary Table 1). FM 9160 B2F had the lowest rating (1.88), while ST 4288 B2F had the lowest percentages of infected (50.5 %) and defoliated leaves (14.6 %) with a rating of 2.00, followed by MD 25ne (2.06) and PHX 4912 WRF (2.07). DP 1048 B2RF had the highest leaf severity rating (3.32) and highest percentages of infected leaves (80.7 %) and defoliated leaves (51.6 %).

In 13Test 1—Trial NV (Supplementary Table 2), the leaf severity rating and percentages of infected leaves and defoliated leaves at 38 DAI were significantly different among 32 cultivars at $p = 0.10$ level, while the percentage of infected plants did not show significant differences indicating a relatively uniform inoculation (89–100 % plants infected except for one genotype). Four cultivars (FM 9170 B2F, Nitro 44 B2RF, NG 4010 B2RF, and DP 1219 B2RF) were VW resistant, with leaf ratings ranging from 1.21 to 1.89 and infected leaves from 22.8 to 42.0 %, while 11 cultivars (PHY 367 WRF, Dinero B2RF, PHX 3122 WRF, PHX 4339-06 WRF, PHY 565 WRF, CR 103233 B2RF, BX 1348GLB2, AM 1511 B2RF, PHY 499 WRF, PHX 3074 WRF, and PHX 4339-CB WRF) were most susceptible to VW with leaf severity ratings from 3.00 to 3.72 and infected leaves from 65.6 to 76.3 % (Supplementary Table 2). At 67 DAI, the stems of plants were dissected to assess stem severity based on vascular discoloration. Among the 32 cultivars, the stem severity rating was not significantly different, while the percentage of infected plants showed significant differences (Supplementary Table 2). Some plants with leaf symptoms did not show vascular discoloration, and were therefore

classified as non-infected. Most of the resistant cultivars, as evaluated previously based on the visual rating of leaf symptoms, had lower percentages of infected plants based on vascular symptoms.

In 13Test 3—Trial HQ, all four VW resistance parameters based on leaves (i.e. the leaf severity rating and the percentages of infected plants, infected leaves, and defoliated leaves) were significantly different among 32 cultivars at 38 DAI (Supplementary Table 3). Although seedlings in this test had relatively lower leaf severity ratings, seven resistant cultivars (FM 9058F, MD 10-5, All-Tex Nitro 44B2 RF, LA 17, FM 2484 B2F, DP 1032 B2RF, and Ark 0410-21) were most resistant with leaf severity ratings ranging from 0.64 to 1.28. ST 4145 LLB2 was most susceptible with the highest leaf severity rating of 2.92 (Supplementary Table 3). However, the stem severity rating and percentage of infected plants were not significantly different among the 32 cultivars based on vascular symptoms at 52 DAI (Supplementary Table 3).

Evaluation of VW resistance in Pima cotton

The leaf severity rating and percentages of infected and defoliated leaves at 41 DAI were significantly different among ten Pima cotton cultivars and lines tested in 11Test 3—Trial B (Supplementary Table 4). The percentage of infected plants in each of the Pima genotypes was 100 % with no escapes to infection by *V. dahliae*, indicating the success of double inoculation. The leaf severity rating for Pima PHY 800, Pima PHY 830, and Pima DP 357 was lower than 2.00, showing higher levels of VW resistance. These three cultivars also had lower percentages of infected and defoliated leaves. However, the leaf severity ratings for Pima S-7, 06E2032-1 and 06E2023-1 were 3.08, 3.33 and 3.46, respectively, making them the most susceptible Pima genotypes to VW in this test.

Evaluation of VW resistance in elite breeding lines from the US public cotton breeding programs

In 11Test 2—Trial RB, differences in leaf severity rating at 41 DAI were significant among the 34 genotypes tested (Supplementary Table 5). TAM 03WZ-37 had the lowest percentages of infected and defoliated leaves and the lowest leaf severity rating,

followed by MD 25ne and PD 05041, while PX 03202-65-1 and DP 393 were most susceptible to VW with leaf severity ratings of 3.25 and 3.37, respectively.

In 13Test 2—Trial RB, the leaf severity rating and percentages of infected plants and infected leaves at 38 DAI were also significantly different among 32 advanced breeding lines, while the percentage of defoliated leaves was not significantly different. NC 11AZ01, NM 11Q1157, SG 105, MD 10-5, and Ark 0403-3 were most resistant, with ratings ranging from 1.06 to 1.26, while GA 2008057, Ark 0409-17, and PD 05071 were the most susceptible lines with a leaf severity rating of 2.83, 2.89, and 3.17, respectively (Supplementary Table 6). At 52 DAI, the disease severity was assessed again based on vascular discoloration of stems. Although different methods were used to evaluate VW resistance at different cotton growth stages, the results from the two screening methods showed a similar tendency.

VW resistance in advanced breeding lines derived from Upland × Pima

In 12Test 2—Trial H, the leaf severity rating ranged from 1.47 to 4.87, showing significant differences among the 32 genotypes tested (Supplementary Table 7). Specifically 19 lines and the Upland parent Acala 1517-99 had significantly higher leaf severity rating than the Pima parent Pima PHY 76. However, 08N1618 had the lowest rating (1.8) among the 29 introgression lines, followed by several other lines with higher ratings but insignificantly different from the Pima parent. In 12Test 1—Trial G and 12Test 3—Trial K, the disease rating was not significantly different among the 32 genotypes tested in each test, and both parents had ratings higher than 3.30 (Supplementary Table 7). Among the three tests, the common check Acala 1517-08 and the Upland parent Acala 1517-99 performed consistently with ratings from 3.1 to 4.1, while the Pima parent PHY 76 showed susceptibility to VW in two (with severity ratings of 3.3 and 4.2) of the three tests, as expected (Zhang et al. 2012). Unexpectedly, it showed a relatively lower disease severity rating (1.5) in one test (12Test2—Trial H). Although genotype × test interaction may exist, the actual reason for the discrepancy was unknown.

Consistency in evaluation of VW resistance between tests and selection of VW resistant genotypes across tests

Based on VW resistance performance of the three tests in 2011 (i.e. 11Test 1—Trial HQ, 11Test 2—Trial RB, and 11Test 3—Trial B), 19 cultivars, which were either resistant or moderately resistant, were chosen to verify their VW resistance. Based on the ANOVA analysis (Supplementary Table 8), although the percentage of infected plants was significantly different among the 19 cultivars perhaps to a single inoculation, the differences in leaf severity rating were not significant, as expected for the selected VW resistant genotypes. The results were in general consistent with those obtained earlier in this study. Even though high severity ratings for some genotypes were observed in all other tests with susceptible ones, inclusion of highly susceptible genotypes in this retest would be preferred.

In this study, 30 cultivars and lines were tested more than once in two entries under different names in the same test, or in different years or tests. Most of them (22) showed consistent VW resistance performance in different tests; however, eight cultivars showed conflicting performance. For example, PHY 375 WRF, the most popular Upland cotton cultivar planted in the US in 2011 (USDA-NASS 2011), was tested four times in different greenhouse tests in 2011 and 2013, but showed different results with leaf severity ratings of 1.47 in two tests, and 2.69 and 3.21 in other two tests. The other seven cultivars had leaf severity ratings at 1.08–1.76 in one test and 2.89–3.32 in another. These results showed that, even under the greenhouse conditions, environmental effects may greatly influence the resistance performance of cotton cultivars due to genotype × environment interactions.

Based on the tests from 2011 to 2013, 17 cultivars, with leaf severity ratings ranging from 1.06 to 2.08, were identified as the most resistant genotypes, while 24 cultivars were most susceptible, with leaf severity ratings ranging from 2.78 to 3.76 (Table 1).

Correlation analysis between VW resistance traits

In the three tests conducted in 2011, i.e. 11Test 1—Trial HQ, 11Test 2—Trial RB, and 11Test 3—Trial B, the correlation analysis indicated that the three resistance traits (leaf severity rating, and percentages of infected leaves and defoliated leaves) were significantly and positively correlated (at $p < 0.0001$ level) with each other in each of these three tests (Table 2).

Similarly, In the three tests of 2013 (13Test1—Trial NV, 13Test2—Trial RB, and 13Test3—Trial HQ), the above three parameters and percentage of infected plants based on leaf symptoms were positively and significantly correlated with each other, except for the correlation between percentage of defoliated leaves with percentage of infected leaves in two tests (Table 3). Once again, the leaf severity rating and percentage of infected leaves had the highest correlation. For evaluation based on vascular discoloration in the stem, significant correlation was detected between stem severity rating and percentage of infected plants in two tests.

However, results varied for correlation between VW resistance traits based on foliar symptoms and vascular discoloration of stems. In 13Test1- Trial NV, the percentage of infected plants based on vascular discoloration of stems was significantly and positively correlated with the four parameters based on leaf symptoms, while the stem severity rating based on stem evaluation did not (Table 3). In 13Test 2—Trial RB, almost the reverse was true in that the two VW resistance traits based on stem vascular discoloration were only significantly correlated with the percentage of infected leaves or disease severity rating based on leaf symptoms (Table 3). However, in 13Test 3—Trial HQ, all the six measurements of VW resistance traits, four criteria based on leaf symptoms and two criteria based on stem symptoms, were positively and significantly correlated with each other (Table 3).

Estimates of heritabilities for VW resistance

The results are shown in Table 4. The heritability for some parameters was not estimated due to insignificant genotypic variation based on ANOVA. For seven of the ten tests with significant genotypic variation for one or more VW resistance parameters, the broad-sense heritabilities ranged between 0.58 and 0.80 with an average of 0.67. The moderate to high heritabilities indicated that the majority of the variation in VW resistance in different tests of this study was due to genetic factors.

Table 1 The most Verticillium wilt (VW) resistant and most susceptible cultivars in 223 cultivars and advanced breeding lines tested in the greenhouse, Las Cruces, 2011–2013

Cultivar	Species	Type/source	Leaf severity rating	Test
Most resistant cultivars or lines				
Nitro 44 B2RF	*G. hirsutum* (AD1)	All-Tex	1.21	T3[a] and T5 (1.28, 1.13)[b]
NG 4010 B2RF	*G. hirsutum* (AD1)	Americot	1.78	T3
DP 1219 B2RF	*G. hirsutum* (AD1)	Delta and Pine Land	1.89	T3 and T5 (1.89, 1.88)
FM 9170 B2F	*G. hirsutum* (AD1)	FiberMax, Bayer CropScience	1.73	T1 and T3 (2.25, 1.21)
FM 9160 B2F	*G. hirsutum* (AD1)	FiberMax, Bayer CropScience	1.91	T1 and T5 (1.88,1.93)
PD 05041	*G. hirsutum* (AD1)	USDA-ARS, SC	2.04	T2
NC 11AZ01	*G. hirsutum* (AD1)	North Caroline State Univ.	1.06	T4
NM 11Q1157	*G. hirsutum* (AD1)	New Mexico State Univ.	1.51	T5 and T4 (1.94, 1.07)
08N1618	*G. hirsutum* (AD1)	New Mexico State Univ.	1.80	T8
MD 10-5	*G. hirsutum* (AD1)	USDA-ARS, Stoneville, MS	1.13	T5 and T4 (1.08, 1.18)
Ark 0403-3	*G. hirsutum* (AD1)	University of Arkansas	1.26	T4
MD 25ne	*G. hirsutum* (AD1)	USDA-ARS, Stoneville, MS	2.05	T1 and T2 (2.06, 2.04)
ST 4288 B2F	*G. hirsutum* (AD1)	Stoneville Pedigreed, Bayer CropSceince	2.00	T1
Pima COBALT	*G. barbadense* (AD2)	Bayer CropScience	2.08	T6
Pima DP 357	*G. barbadense* (AD2)	Delta and Pine Land, Monsanto	1.97	T6
Pima PHY 800	*G. barbadense* (AD2)	Phytogen, Dow AgroScience	1.29	T6
Pima PHY 830	*G. barbadense* (AD2)	Phytogen, Dow AgroScience	1.83	T6
Most susceptible cultivars or lines				
Dinero B2RF	*G. hirsutum* (AD1)	All-Tex	3.11	T3
CR 103233 B2RF	*G. hirsutum* (AD1)	All-Tex	3.33	T3
AM 1511 B2RF	*G. hirsutum* (AD1)	Americot	3.44	T3
BX 1348 GLB2	*G. hirsutum* (AD1)	Bayer CropScience	3.40	T3
DP 1050 B2RF	*G. hirsutum* (AD1)	Delta and Pine Land, Monsanto	3.06	T1
DP 555 BG/RR	*G. hirsutum* (AD1)	Delta and Pine Land, Monsanto	2.83	T1 (2.48, 3.17)
DP 1043 B2RF	*G. hirsutum* (AD1)	Delta and Pine Land, Monsanto	3.19	T1
PD 05071	*G. hirsutum* (AD1)	USDA-ARS, SC	3.17	T4
PHY 367 WRF	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	3.05	T1 and T3 (3.09, 3.13)
PHY 72	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	2.85	T1 (2.58, 3.11)
PX 03202-65-1	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	3.25	T2
PHY 565 WRF	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	2.78	T1 and T3 (2.26, 3.30)
PHY 499 WRF	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	3.04	T1 and T3 (2.50, 3.58)
ST 4145 LLB2	*G. hirsutum* (AD1)	Stoneville Pedigreed, Bayer CropScience	2.95	T3 and T5 (2.98, 2.92)
ST 4554 B2RF	*G. hirsutum* (AD1)	Stoneville Pedigreed, Bayer CropScience	3.18	T1
MD 10	*G. hirsutum* (AD1)	USDA-ARS, Stoneville, MS	3.10	T1
PHX 3122 WRF	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	3.16	T3
PHX 4339-06 WRF	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	3.17	T3
PHX 3074 WRF	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	3.66	T3
PHX 4339-CB WRF	*G. hirsutum* (AD1)	Phytogen, Dow AgroScience	3.72	T3
Acala 1517-99	*G. hirsutum* (AD1)	New Mexico State University	3.86	T7, T8 and T9
Acala 1517-08	*G. hirsutum* (AD1)	New Mexico State University	3.13	T7, T8 and T9

Table 1 continued

Cultivar	Species	Type/source	Leaf severity rating	Test
NM 06E2023-1	*G. barbadense* (AD2)	New Mexico State University	3.46	T6
Pima S-7	*G. barbadense* (AD2)	USDA-ARS, Maricopa, AZ	3.08	T6

[a] *T1* 11Test 1—Trial HQ, *T2* 11Test 2—Trial RB, *T3* 13Test 1—Trial NV, *T4* 13Test 2—Trial RB, *T5* 13Test3—Trial HQ, *T6* 11Test 3—Trial B, *T7* 12Test 2—Trial G, *T8* 12Test 2—Trial H, *T9* 12Test3—Trial K

[b] The number in parenthesis means the leaf severity rating in each test if cultivars were evaluated repeatedly

Table 2 Coefficient of correlation of Verticillium wilt resistance traits within each test, 2011

Test	Traits	Leaf severity rating	Infected leaves (%)	Defoliated leaves (%)
11Test 1: Trial HQ	Rating	1.00		
	Infected leaves (%)	0.87***	1.00	
	Defoliated leaves (%)	0.65***	0.59***	1.00
11Test 2: Trial RB	Rating	1.00		
	Infected leaves (%)	0.91***	1.00	
	Defoliated leaves (%)	0.64***	0.62***	1.00
11Test 3: Trial B	Rating	1.00		
	Infected leaves (%)	0.87***	1.00	
	Defoliated leaves (%)	0.58***	0.61***	1.00

*** Significant at the 0.001 level

Discussion

VW resistance performance among 136 commercial cultivars and advanced breeding lines

In this study, ten greenhouse tests were conducted to screen commercial Upland cotton cultivars and elite breeding lines for VW resistance. The cultivars developed from seed companies and the elite lines from the US public breeding programs were submitted each year for official variety tests or high quality tests in different states. Therefore, there were a few of them in common in more than one test. Of 136 cultivars and lines, 32 cultivars showed relatively higher levels of resistance (leaf severity rating <2.1) against VW, accounting for 23.5 % of the tested genotypes, while 36 cultivars showed a leaf severity rating of 3.0 or

greater, accounting for 26.5 % of the tested genotypes. In fact, some of the cultivars displayed similar resistance performance as observed in field tests by Wheeler and Woodward (2010, 2012) in Texas. For instance, Nitro 44 B2RF, FM 9160 B2F, ST 4288 B2F, NG 4010 B2RF, FM 9160 B2F, and FM 9170 B2F were resistant to VW; DP 1044 B3F, FM 9058 F, DP 1032B3RF showed tolerance to VW; and DP 1050B2RF, PHY 367 WRF, DP 161B2RF, PHY 375 WRF, and DP 1048B2RF were susceptible to VW (Wheeler and Woodward 2010, 2012).

The major Pima cultivars, Pima PHY 830, Pima PHY 800, and Pima DP 360, which accounted for 14.5 % of Pima acreage in the US in 2011 (USDA-NASS 2011), showed resistance to VW in the current tests. These cultivars and other VW resistant Upland cotton cultivars identified in the current study are a good choice to be grown in VW infected fields. They can be also used as resistance sources for breeding for VW resistant cultivars.

Inoculation methods and evaluation parameters in screening for VW resistance

Concentration of inoculum, pathotypes of isolates, plant genotype, and environmental factors, such as temperature, humidity, plant density, and even the amount and the type of fertilizers could influence the development of VW (Paplomatas et al. 1992). Both field and greenhouse tests have been extensively used to select VW resistant sources. Field tests can reflect the natural conditions and identify adult plant resistance to VW. But, field results may be influenced by the lack of uniform inoculum and varying environmental conditions. Greenhouse inoculation is a good alternative method to screen cotton for resistance to VW, because inoculation can be conducted under uniform and optimal environment, and young seedlings can be evaluated. Greenhouse studies not only

Table 3 Correlation analysis of six Verticillium wilt resistance traits in three tests, 2013

Test	Leaf severity rating[a]	Infected plants (%)[a]	Infected leaves (%)[a]	Defoliated leaves (%)[a]	Stem severity rating[b]	Infected plants (%)[b]
13Test 1: Trial NV						
Leaf severity rating[a]	1.00[c]					
Infected plants (%)[a]	0.51**	1.00				
Infected leaves (%)[a]	0.92***	0.36*	1.00			
Defoliated leaves (%)[a]	0.75***	0.15	0.75***	1.00		
Stem severity rating[b]	0.03	0.13	0.15	0.03	1.00	
Infected plants (%)[b]	0.73***	0.38*	0.79***	0.46**	0.14	1.00
13Test 2: Trial RB						
Leaf severity rating[a]	1.00					
Infected plants (%)[a]	0.46**	1.00				
Infected leaves (%)[a]	0.87***	0.47**	1.00			
Defoliated leaves (%)[a]	0.93***	0.40*	0.78***	1.00		
Stem severity rating[b]	0.39*	0.26	0.47**	0.29	1.00	
Infected plants (%)[b]	0.27	0.14	0.38*	0.14	0.76***	1.00
13Test 3: Trial HQ						
Leaf severity rating[a]	1.00					
Infected plants (%)[a]	0.46**	1.00				
Infected leaves (%)[a]	0.93**	0.51**	1.00			
Defoliated leaves (%)[a]	0.89***	0.26	0.80***	1.00		
Stem severity rating[b]	0.58***	0.41**	0.49**	0.48**	1.00	
Infected plants (%)[b]	0.61***	0.47**	0.64***	0.40*	0.44**	1.00

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively

[a] Evaluation at 38 DAI using symptoms of leaves

[b] Evaluation at 52/67 DAI by using symptoms of stem

[c] Correlation coefficient (r)

eliminate or minimize the influence from variable environmental factors, but they can also save space and speed the process of disease resistance evaluation (Devey and Rosielle 1986). Good correlations were also observed between greenhouse and field evaluations for resistance to VW (Wilhelm et al. 1974).

As the present study demonstrated, genotypic variation in VW resistance can be detected in greenhouse screening based on analysis of variance on replicated tests, and most of selected resistant genotypes were shown to be resistant when retested. In another greenhouse study with 378 Upland cotton accessions (Zhou 2013), VW resistance traits were significantly and positively correlated between the two tests conducted; however, significant genotype × test interactions were also detected. Therefore, as with studies on other quantitative traits, screening for VW resistance should be performed in more than one replicated test. However, a genotype with a high VW severity rating in a single greenhouse or field screening is not necessary to be retested and can be considered susceptible to VW. However, genotypes with low leaf severity ratings may be due to an overall low level of inoculum or escapes from infection by *V. dahliae* and should be retested to confirm VW resistance. In all cases, a susceptible genotype and a resistant genotype with known and consistent levels of VW responses should be included as controls to gauge the success of a greenhouse or field screening.

The stem-puncture method was previously used to introduce VW pathogen into cotton plants (Bolek et al. 2005; Göre et al. 2009). Using the same cultivars, Devey and Rosielle (1986) conducted VW resistance evaluation both in the greenhouse and in the field. They found that the stem inoculation achieved more consistent results with the resistance performance

Table 4 Estimate of broad-sense heritabilities of Verticillium wilt resistance traits based on the results of ANOVA

Test	Leaf severity rating	Infected plants (%)	Infected leaves (%)	Defoliated leaves (%)
11Test 1: Trial HQ	0.58			0.59
11Test 2: Trial RB	0.61			
11Test 3: Trial B	0.79		0.80	0.75
12Test 1: Trial G				
12Test 2: Trial H	0.65			
12Test 3: Trial K				
13Test 1: Trial NV	0.60		0.62	0.70
13Test 2: Trial RB	0.64	0.68	0.67	
13Test 3: Trial HQ	0.74	0.64	0.75	0.60

Heritability: Based on ANOVA, the broad-sense heritabilities were estimated using the following formula: Heritability = MS(genotype)/ [MS(genotype) + MS(error)]

evaluation in the field than a root inoculation method. However, stem inoculation method undermines the interpretation of the resistance mechanisms compared to root inoculation. The interaction between the pathogen and the plant on the root surface of host tissue is bypassed by stem inoculation. Moreover, this method is time consuming and difficult to use with young seedlings. Stem-inoculation could not distinguish resistant cultivars from susceptible ones in olive, *Olea europaea* (Cirulli et al. 2008). In the present study, root inoculation was achieved by pouring the inoculum directly onto the soil surface in a pot containing ∼5 plants. Furthermore, utilization of the double inoculation method eliminated escapes and ensured plant infection by the pathogen.

In the three tests conducted in 2013, VW severity estimated based on foliar symptoms proved to be more reliable than the evaluation based on stem vascular discoloration. This result is consistent with a previous study (Marani and Yaacobi 1976). In addition, screening cotton for VW resistance based on vascular discoloration requires dissecting stems longitudinally. Therefore, it is more time-consuming and labor intensive than the foliar symptom evaluation. Furthermore, it also introduces more experimental errors. A plant without vascular discoloration of the stem may still show leaf symptoms, as observed in this study. Four parameters, i.e. leaf severity rating and percentages of infected plants, infected leaves and defoliated leaves, were used to assess VW resistance. In most

tests, the four parameters were found to be significantly and positively correlated with each other, indicating that the VW resistance traits are interrelated. However, the coefficients of variation (CV) differed among these four VW resistance traits. The CV for the percentage of infected plants was understandably the lowest ranging from 3 to 12 %, due to double inoculations for the goal to avoid escapes. It was followed by the CV for the percentage of infected leaves (20–29 %) and the leaf severity rating (23–59 %). The highest CV (25–119 %) was from the percentage of defoliated leaves. However, in most cases, the percentage of infected plants was not significantly different among tested cultivars and lines, indicating that uniform inoculation was achieved for the tests. In fact, the percentage of infected plants was even up to 100 % in some tests. Hence, unlike the field evaluation where percentage of infected plants can be used for screening plant resistance against VW (Zhang et al. 2012), in greenhouse tests percentage of infected plants was not a good parameter to assess VW resistance. The percentage of defoliated leaves may not be suitable to assess disease severity due to its highest CV and disregard of symptomatic leaves.

Leaf disease severity rating is subjective and is hard to use to distinguish two neighboring ratings in some cases, but it is fast and convenient to use. Furthermore, significant genotypic variation in VW resistance based on ratings was detected in seven of the nine tests. These advantages, together with its relatively lower CV and high correlations with the percentage of infected leaves, make leaf severity rating a choice of the parameter for screening cotton for VW resistance. On the other hand, the percentage of infected leaves including defoliated leaves is objective and can accurately quantify plant responses to the VW disease, but counting leaves is a time-consuming and laborious process. Furthermore, leaf growth is also affected by other genetic factors. In addition, significant genotypic variation in this parameter was detected in only four of the nine tests in this study. Therefore, leaf severity rating remains the choice of parameter in screening cotton for VW resistance. However, based on our experience, deciding between two severity ratings for some plants could be difficult due to the subjective nature of the parameter. In addition to leaf severity rating, the percentage of infected leaves may be used in some instances especially in genetic mapping studies of VW resistance.

In summary, we screened 84 current commercial cultivars from major US seed companies, 52 advanced breeding lines from the US public breeding programs, and 87 introgression lines from a cross between Acala 1517-99 × Pima PHY 76 for VW resistance. Among 10 separate replicated tests (each contained different genotypes) in the greenhouse, significant genetic variation (accounting for an average of 67 % of phenotypic variation) in VW resistance was detected in seven tests. This resulted in the identification of six Upland cultivars, five advanced lines, two introgression lines from Upland × Pima, and four Pima cultivars with higher levels of VW resistance. Based on our experiences, a successful greenhouse screening for VW resistance in cotton is dependent on the following factors:

1. Uniform seedling emergence: 6–8 seeds per pot are sown in 4-in plots filled with a commercial potting soil to ensure 4–5 uniform seedlings per pot.
2. Avoidance of escapes from infection by double inoculations, with the first inoculation performed at the second true leaf stage followed by another inoculation within a week using a defoliating-type isolate of *V. dahliae*.
3. Maintain temperature within 20–28 °C.
4. Maintain soil moisture at field capacity through daily irrigation.
5. Experimental design: a randomized complete block design should be used with 3–4 replications and 5–10 plants per replication for each genotype. Always include a common susceptible control and a common resistant control in each test.
6. VW severity screening: a month after the first inoculation, plants can be screened for plant responses to VW infection. Leaf severity rating can be used for a large scale germplasm screening because of its simplicity, rapidness, relatively low coefficient of variation, and a high resolution to differentiate resistant genotypes from susceptible ones. For a more quantitative assessment in some cases, percentage of infected leaves may be used.

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