Growth Response of Defoliating (D) and Non-Defoliating (ND) Strains of *Verticillium dahliae* Under Temperature Stress Support Suspected Differences in their Temperature Tolerance and Niche Expansion Capability.

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ABSTRACT

Temperature has huge influence on niche expansion capability of Verticillium dahlia. Phenotypic differences were observed between defoliating (D) and non-defoliating (ND) of V. derhliae at 25ºC, 28ºC and 32ºC. We conducted temperature stress response test on ten test (10) strains of V. dahliae consisting of five defoliating (HB-19, HB-10, HB-42, XJ28, XJ-36), five non-defoliating (D08047, D08165, XJ-14, XJ-02, XJ-63) pathotypes respectively. A 1.0µl of fungal spores was aseptically introduced into a PDA plate amended with kanamycin, data on radial growth were recorded at four days intervals for five times. Temperature had visible influence on mycelial growth of V. dahliae at all the temperatures tested, mean growth response decreases above and below 25ºC as optimum growth temperature followed by 20ºc, then 28ºC as third. At 28°C treatment, D-strains recorded significantly higher growth (highest HB42 = 48.27mm) compared to ND-strains (highest D08165 = 42.33 mm). There is no significant variation in mean growth responses within the D-strains, but there is significant intragroup variation in the mean growth responses among the ND-strains at 28°C. While at 30°C maximum mean growth among the D-strains was recorded by HB-19 (40.333mm), with XJ-36 (36.93mm), while among the ND-strains XJ-02 (26.93 mm) recorded highest mean growth. All the D-strains showed significantly higher temperature tolerance at 30°C compared to ND-strains. PCR was conducted using Primer pairs specific for Defoliating, Non-defoliating; Race 1, Race 2; and MATE-1-1-1, MATE-1-2-1 at C1000 Touch™ Thermocycler (BIORAD 2014-2015) to determine the genotypes of the test strains. Amplified fragments were run on 1% gel in 1 X TAE buffer along with DNA marker iii ladders, viewed under UV light in molecular imager® Gel Doc™ XR imaging system (BIO-RAD Universal Hood ii) and photographed. Both 5 D-strains were confirmed to be the defoliating by the PCR, so also the 5 ND-strains as Non-defoliating strains.

Keywords: Verticillium dahliae, Defoliating and Non-defoliating, temperature stress, Melanization, Niche expansion capability.

INTRODUCTION

Microbes are constantly faced with sudden environmental changes that sometimes end up compromising their survival and pathogenic ability. To summon stresses, microbes are constantly evolving various sensing mechanisms and signal transductions systems that promote their adaptation to new challenges through metabolism, cell cycle progress, membrane architecture, proteostasis, etc. (Requenna. et al., 2012).

Temperature stress can have both positive and negative impacts, decreasing plant stress during the cold seasons and increasing plant stress during warm seasons (Walther et al., 2002; Ahuja et al., 2010). The increased temperature could also promote symptoms such as wilting, leaf folding, and changes in the synthesis of proteins,

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enzymes, and other compounds, which could increase susceptibility to pathogens (Foden et al., 2008; Stillman, 2009).

MATERIALS AND METHODS

Fungal Isolates, Media and Culture Conditions

Ten test strains of Verticillium dahliae consisting of six defoliating (HB-19, HB-10, HB-42, XJ28, XJ-36) and six non-defoliating (D08047, D08165, XJ-14, XJ-02, XJ-63) pathotypes respectively, were used in this study. The isolates were supplied by the microbial culture library section of the laboratory of cotton disease, institute of food science and technology, maintained under the supervision of Prof. Dai Xiao-feng, Institute of Food Science and Technology (IFST), Chinese Academy of Agricultural Sciences. All isolates were first re-activated, a single colony from each isolate was mass cultured on CM without antibiotic amendment, spores were preserved in 50% Glycerol, stored at -80℃ as back up.

Reactivation of test isolates and spore production

In order to study the in vitro growth response of ten test strains of V. dahliae under temperature stress, experimental isolates were first revived by plating on PDA amended with 10% v/v of kanamycin, and Amphicilin (400µm/400lm). Single colonies from above PDA cultures at 5-6 days post inoculation (PI) were then aseptically transferred and mass cultured in complete medium (C.M) in triplicates for each isolate, placed under shaker incubator at 25^{o}C for four days.

Evaluation of in vitro growth response of Verticillium dahliae

A 1.0µl of the spores from (2.4.1) above was aseptically introduced into a PDA plate, amended with kanamycin. Sealed culture plates were then incubated at their respective temperature condition (15^{o}C, 20^{o}C, 25^{o}C, 28^{o}C 30^{o}C and 32^{o}C) with three replications for each isolate. Data on radial growth were recorded at four days intervals five times. Phenotypes were photographed at the end of the experiment using a CANON EOS 70D camera.

The experiment was laid in a factorial design with the temperature and isolates as factors, and the Petri dishes as replication. Two way analysis of variance (ANOVA) was conducted using IBM SPSS Statistics v20-64 bit.

Growing and maintaining of Stock culture

All isolates used were single-colony purified and maintained as conidia suspensions at 28℃ in glycerol diluted by half strength with sterile dd water (50% glycerol vol/vol), deposited with the microbial culture collection section of this laboratory and could be retrieved new for on-demand. Cultures were grown on the following media for phenotyping and on CM for DNA isolation.

Identification of Genotypes of the Test Strains:

Fungal tissue for DNA extraction

To provide fungal tissue for genotyping the test strains, a single colony of each test isolate was aseptically cultured in CM without antibiotic and incubated at 25℃ for five days under shaker incubation. Tissue was harvested under centrifuge at 12 rpm for 4 min, wrap up in Reynolds foil paper, lyophilized in liquid nitrogen to remove moisture and stored at -80℃.

DNA extraction

Total fungal DNA was extracted from each of the lyophilized tissue using TIANGEN DNA for plant kit following the manufacturer’s protocol.

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Polymerase chain reaction (PCR)

PCR was conducted using the Primer specific for the Defoliating, Non-defoliating; Race 1, Race 2; and MATE-1-1-1, MATE-1-2-1 at C1000 Touch™ Thermocycler (BIORAD 2014-2015) under the following condition:

A 25 µl PCR reaction mixture containing 12.5 µl of 2x EasyTaq® PCR SuperMix, 9.5 µl nuclease-free water, 1.0 µl each of primer pair (F/R) and 1 µl of DNA template was prepared for each sample. The reaction conditions were initialization at 95℃ for 5min, followed by denaturation at 95℃ for 0:30 sec, annealing at 60℃ for 0:30 sec., extension or elongation at 72℃ for 30sec, followed by final extension or elongation at 72℃ for 10:00 min and finally hold at 4℃ for 0:00 (∞) [GOTO 2, 30X; Lid at 105℃]. PCR reaction performed, as mentioned in (Table 1.0).

Table 1. Components for Pcr Reaction

Reaction component of the PCR	
Components	**Volume (µl)**
	(1X)
2x EasyTaq® PCR Supermix	12.5
Primer F (10µM)	1
Primer R (10µM)	1
DNA Template (30ng/µl)	1
Nuclease free H2O	9.5
Total Volume (µl)	25

PCR conditions:

94ºC-5 min

94ºC-30 sec

54ºC-30 sec

72ºC-1 min 30 sec

72ºC-10 min

Amplified fragments were run on 1% gel in 1 X TAE buffer along with DNA marker iii ladders, viewed under UV light in molecular imager® Gel Doc™ XR imaging system (BIO-RAD Universal Hood ii) and photographed.

Pathotypes specific primes used:

1-DF (CATGTTGCTCTGTTGACTGG),

1-DR (GACACGGTATCTTTGCTGAA), gives nearly 500bp, annealing temperatures of 60℃,

2-NDF (CAGGGGATACTGGTACGAGACG),

2-NDR (ATGAGTATTGCCGATAAGAACA), gives 1500bp, are specific (Pérez-Artés, et al., 2000).

Race-specific Primer pairs:

1-VdAve1F (AAGGGGTCTTGCTAGGATGG)

1-VdAve1R (TGAAACACTTGTCCTCTTGCT) gives approximately 900 bp, V. dahliae race 1, (de Jonge, et al., 2012)

2-VdR2F (ACTTAACGAAAGCATGCGC)

2-VdR2R (CTTGACTTGCCGGCTCC), gives 260 bp, identified race 2 (Short et al., 2014).

Primer pair specific for Mating type:

1-AlF3 (CGATCGCGATATCGGCAAGG),

1-MAT1–1–1 R (CAGTCAGATCCAACCTGCTGGCC) about a 600-bp fragment of MAT1–1–2-HMG21F (CGGCCGCCCAATTCGTACATCC) (Inderbitzin, et al., 2011a, 2011b; Klosterman, et al., 2011; Short, et al., 2014; Milgroom, et al., 2013)

2-MAT-2-1R (CATGCCTTCCATGCCATTAGTAGCC), amplify approximately 300 bp fragment from MAT1–2–1 isolate as reported in (Usami et al. 2002, 2008; Klosterman, et al., 2011; Inderbitzin, et al., 2011a, 2011b; Short, et al., 2014).

Amplified fragments (14µl) were run on 1% agarose gel electrophoresis, viewed under UV light, and photographed using Molecular imager® Gel Dox (universal Hood ii) Biorad Lab. Inc, C.A.

Phenotypes of the test strains:

Phenotypes were grown on a PDA amended with 10% v/v ampicillin from spores grown in CM above, incubated at 25℃, and photographed after ten days using a camera (CANON EOS 70 D).

RESULTS

Phenotypes and Genotypes of the test isolates used in the study:

Test strains of Verticillium dahliae culture from the library were kindly issued by the microbial culture library section of Prof. Dai Xiaofeng's laboratory of cotton pathology, CAAS, for this study. They were first re-activated to get a single colony and spore produced on CM media from the single colony under the shaker incubator at 25℃. Phenotypes of all the test strains were produced by inoculating 2µ spore on PDA plates and incubated at 25oC for 12 days. Most of the defoliating phenotypes (D) start producing melanin as early as eight to ten days P.I under 25℃ growth condition. In comparison, non-defoliating pathotypes (ND) start melanization around thirteen to fourteen days P.I, under 25oC growth condition. All the phenotypes look truly V. dahliae, and no aerial mycelium observed in both groups (Figure 1a).

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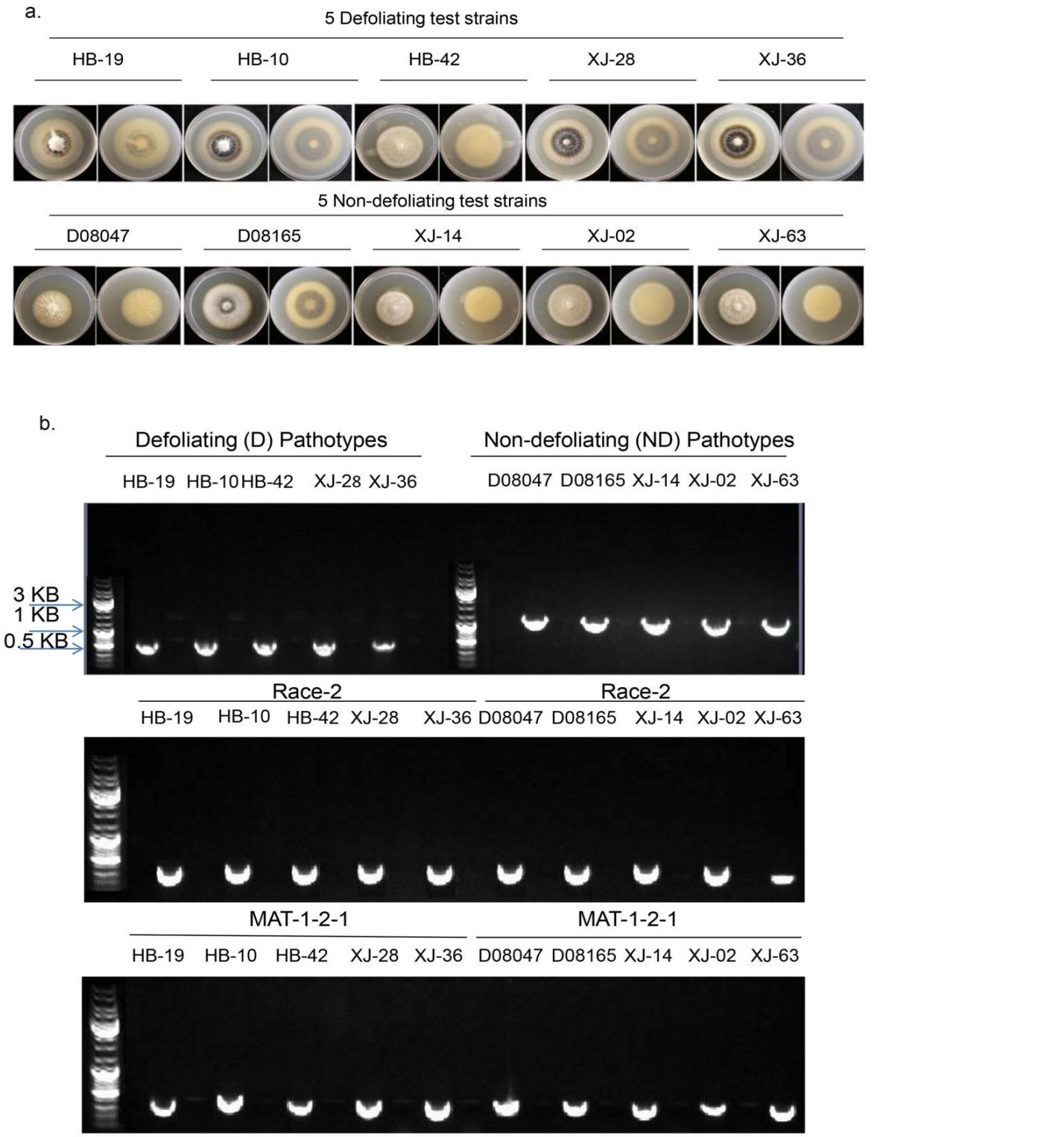

Figure 1. Phenotypes And Gel Bands Displaying Genotypes Of Study Isolates. A Shows Phenotypes Of The Test Isolates Used In The Study Confirmed, Where Up = Face Picture And Down = Bottom Picture Of The Culture Plate And B. Is Gel Bands Displaying Genotypes Of The Test Isolates Used In This Study With Gene Ruler (Name Of The Dna Ladder) At Left Y-Axis.

We also determined the genotypes of all the test strains using PCR protocol, and primers pairs specific to each character were used to amplify the fragments as reported by (Inderbitzin et al., 2013) (Klosterman et al., 2011) and (Short et al., 2014). All the test isolates were analyzed for the detection of pathotypes, races and mating types by the specific PCR assay based on the presence of 500 bp (D pathotype), 1500 bp (ND pathotype), 900 bp (race 1), 260 bp (race 2) and 600-bp (MAT-1-1-1), 300 bp (MAT-1-2-1) amplicons. The first five strains were defoliating pathotypes (D), and other fives were non-defoliating pathotypes (ND), while all the ten strains were race type 2 (R-2). Likewise, all of them were mating types 2 (MAT-1-2-1), as shown in (Figure 1b).

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Defoliating and Non-Defoliating isolates displayed similar growth pattern at 20ºC and 25ºC:

The ability to grow at a certain temperature range is universal among plant pathogens. In Ascomycetes, the transition from a saprophyte or dormant resting structure into a plant pathogen is associated with global changes in gene expression that contribute to the pathogenesis. The temperature had a visible influence on mycelial growth of V. dahliae at all the temperatures tested, with overall decreasing growth responses in both directions from 25ºC (i.e., above and below 25ºC) as shown in (Table 2.0). Analysis of variance has revealed that the interaction between temperature treatment and Verticillium strain significantly influence the colony diameter (or mycelial growth) as shown with wide variation between D and ND in (Table 3).

The mean growth response per isolates varies significant between D and ND, with all D-strains having significantly higher growth response compared to ND-strains. The highest grand growth response (overall performance) in the D-strains was recorded by XJ28 (41.96mm[a]), while the highest in the ND-strains was D080165 (35.49mm[c]) as shown in (Table 2.0). There are intra strains variations in the responses of ND-group with D08165, and XJ-02 ranked as **c**; while XJ-63, XJ-14 both ranked as **d**, and D08047 ranked as **e** (Table 2.0). But little intra strains variation within the D–strains, with XJ28, HB-19; HB-42, and HB-10 strains having the highest growth, and both ranked as **a**, while XJ-36 was ranked **b** (Table 2.0). The overall performance of the test strains can be separated into six subsets base on homogeneity test, with all the three groups containing only ND-strains. In contrast, the fourth and fifth groups are the D-strains, and the D-strains appeared to have significantly better overall performance compared to ND-strains. The better performance of the D-strains might be due to the extreme temperature conditions been more than the number of normal temperature conditions tested (Table 2.0). The cumulative performance of the test strains varied significantly among temperature stress, where the entire test strains have the highest mean growth at 25°C (46.73mmA) followed by (44.42mmB). In contrast, 32°C condition has the lowest mean growth (21.85mm F) as shown in (Table 3).

A number of these changes are temperature-regulated and include the induction of virulence-related genes, examples is Cell wall genes (VdCYP1) reported in (Zhang, et al., 2016) and pectin lyase genes in (Chen et al., 2016). Spadoni, et al. (2014) have reported a general decrease in expressions of two genes involved in fruits ripening (β-Gal, PL, PG & PME) in hot water treated fruits as compared to control after inoculation with Monilinia laxa. In contrast, expression levels of ROS scavenging genes and PA, CHI, HSP70, increased in hot water treated samples compared to untreated fruits after inoculation with Monilinia laxa.

Table 2. Growth performance of each isolate throughout the experiment and the overall performance of all the isolates per temparature level.

V. dahliae_Isolates	Rankking	Least Mean (mm)
XJ28	A	41.96[a]
HB-10	A	41.49[a]
HB-19	A	41.16[a]
HB-42	A	40.69[a]
XJ-36	B	39.16[b]
D08165	C	35.49[c]
XJ-02	C	35.00[c]
XJ-63	D	33.13[d]
XJ-14	D	32.49[d]
D08047	E	31.32[e]
Pvalue		<.0001
Std Error = 0.350327		
Overall performance by temperature		
Level	**Ranking**	**Mean (mm)**
25	A	46.73
20	B	44.42
28	C	42.96

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15	D	36.05
30	E	31.25
32	F	21.85
Pvalue		<.0001
Std Error = 0.271362		

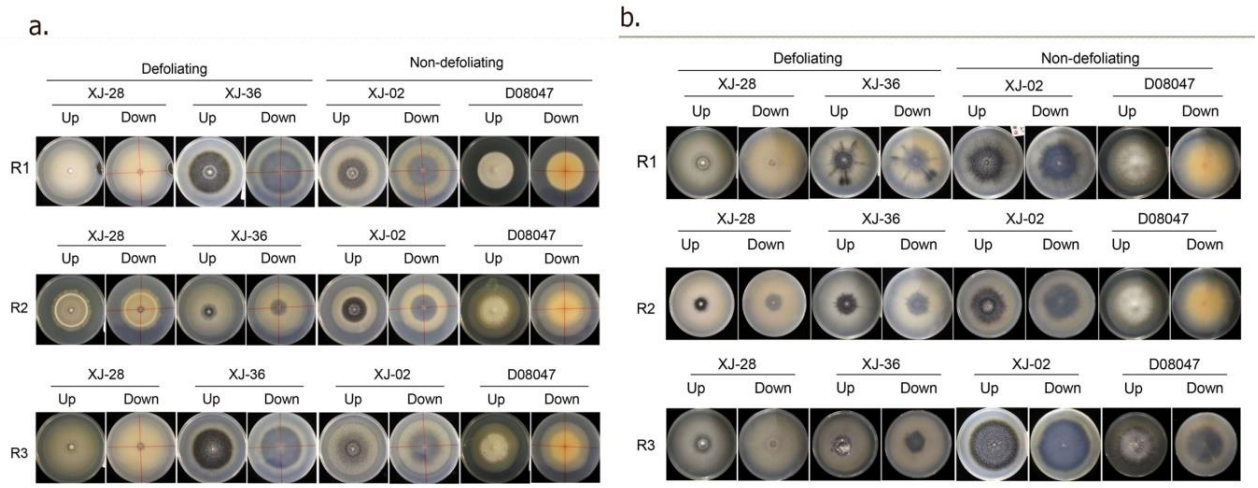

Figure 2 Phenotypes of D and ND of V. dahliae with max and min growth at 20℃ and 25℃; A are phenotypes of D and ND-pathotypes showing their growth response to 20℃ Tm while B demonstrates the phenotypes of D and ND-pathotypes representing their growth response at 35℃. Where R1= replication 1; R2 = replication 2 and R3 = replication 3; Up = face picture and Down = bottom picture of the culture plate/phenotype.

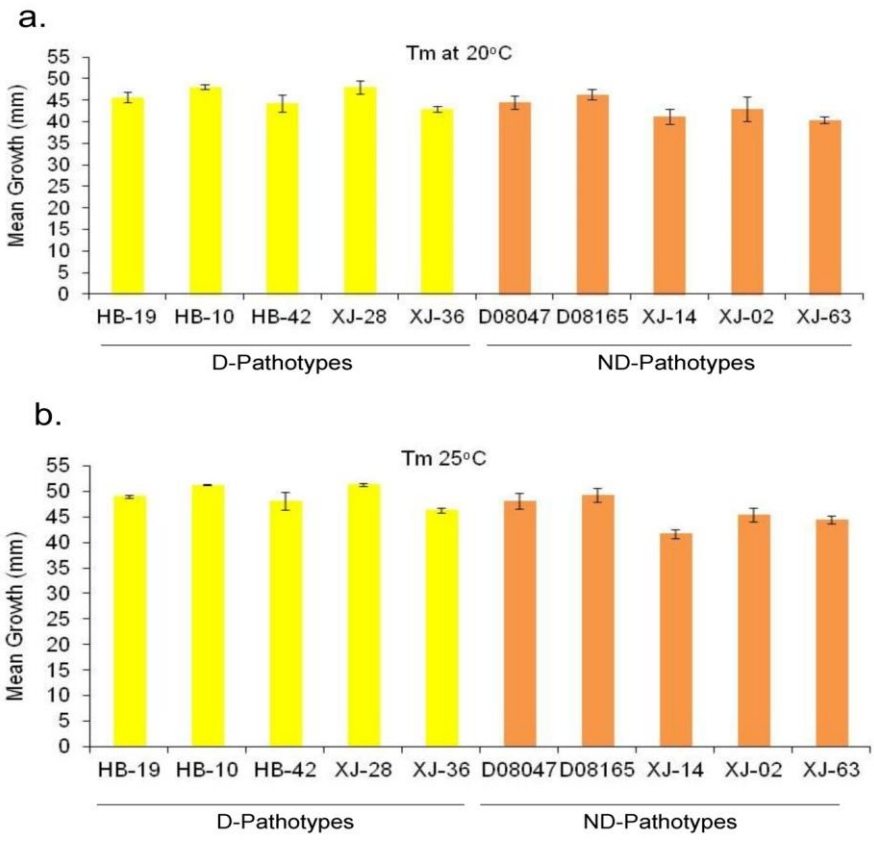

Figure 3 Bar graph depicting mean mycelial growth responses of the ten test isolates at 20℃ and 25℃, Bar graph depicting mean mycelial growth whereas Tm = temperature; Y-axis represents growth response of V. dahlia, and X-axis represents isolates;

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The result of this study indicated that Tm has a profound effect on the growth ability of the entire test isolates (5 D-strains and 5 ND-strains). All the strains have significantly higher growth response at 25℃ temperature compared to all the rest of the condition tested in both D and ND-groups (Table 2.0). There is no significant difference in growth response between D and ND-strains at 20℃ and at 25℃ (Table 3.3 & Table 3.0; Figure 2 and Figure 3). The highest growth response in D-strains at 20℃ was recorded by HB-10 (48.07mm), while the highest growth in ND-strains was recorded by D08165 (46.33mm). And the lowest is XJ-36 (42.93mm) in the D- strains, and XJ-63 (40.40mm) in the ND-strains (Table 3.0; Figure 2A and Figure 3a). While at 25ºC XJ28 (51.33mm) has the highest growth and XJ-36 (46.33mm) recorded the lowest growth among the D-strains, but in the ND-group D08165 (49.33mm) has the highest growth while D08047 (40.33mm) has the lowest growth (Table 3.0; Figure 2b; Figure 3b). Both D-group and ND-group phenotypes both produced dark grey colonies at 21 days P.I with no aerial mycelium, and dark melanin appeared in the mycelial colony after 8-19 days on PDA medium (Figure 2).

At 25ºC XJ28 (51.33 mm) has the highest mean growth response in the D-strains and D080165 (49.33 mm) has the highest mean growth response, but generally, the mean growth responses pattern in the D-strains was not significant compared to ND-strains, and both recorded the highest growth responses at this Tm. In general, mean growth response decreases above and below 25ºC, with a performance at 20ºC showing its second-best optimum temperature followed by 28ºC as third optimum temperature for both pathotypes' strains as can be seen in (Table 3.0). Both temperature, V. dahlia pathotypes, and the interaction of temperature and isolates had significant effects on the growth response, as shown above in (Table 3).

Table 3. Interaction between temperature and V. dahliae isolates growth (mm)

	Tm * Vd Interaction									
	Verticillium dahlia (Vd) Isolates									
Tm (ºC)	XJ28	XJ-36	HB-19	HB-10	HB-42	D08047	D08165	XJ-14	XJ-02	XJ-63
15	39.13a	37.27c	37.73c	38.13b	36.67d	31.13g	34.47f	35.00e	35.40e	35.53e
20	47.93a	42.93c	45.67d	48.07a	44.33b	44.53b	46.33d	41.13e	42.87c	40.40e
25	51.33a	46.33d	49.07b	51.27a	48.13c	40.33h	49.33b	41.67g	45.40e	44.47f
28	47.27b	44.93c	47.13b	47.60b	48.27a	34.93h	42.33d	37.13g	41.13e	38.87f
30	39.00b	36.93c	40.33a	39.13b	39.47b	22.40e	22.80e	23.40ef	26.93d	22.07e
32	28.40a	26.53c	27.00b	24.73d	27.27b	14.60g	17.67e	16.60f	18.27e	17.47e

Std Error = ± 0.858122

The best growth pattern for both D-strains and ND-strains was at 25ºC, while the lowest growth response for both D and ND-strains was at 32ºC; Mean values falling within same letters range or carry the same are statistically insignificant at PValue<0.0001

D-strains of V. dahliae are significantly more tolerant compared to ND-strains

Defoliating pathotypes (D) and non-defoliating pathotypes (ND) were subjected to temperature stress, and after that, their growth response was evaluated. It was observed, that at 15ºC, 28ºC, 30ºC and 32ºC all the colonies from both D and ND were creamy white at the beginning but became packed, stunted and developed a varying degree of melanization (Figure 3.0 a, b, c, & d). D-pathotypes showed early melanin formation but halted at some point of growth. In contrast, melanization formation started latter in ND-pathotypes. Still, it progressed rapidly, covering more than half of the entire colony growth except for D08047, which seems to maintained creamy white colonies at all the temperature tested (Figure 3 a, b, b & b). No aerial mycelial growth was observed from both D-group and ND-group at all the Tm treatments.

Generally, a similar trend in sensitivity to low temperature (15ºC) (decrease in growth) was observed for both defoliation (D), and non-defoliation (ND) pathotypes, with inter, and some intragroup variations (Figure 3a; Table 3.0). The highest mean growth at 15℃ was recorded by XJ28 (39.13 mm), while the lowest mean growth was recorded by D08047 (31.133 mm), as can be seen in (Table 3.0; Figure 3a and Figure 4a). But at 15℃

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growth responses of the D-strains were significantly higher compared to the growth responses of the ND-strains (Table 2.0; Figure 4A). All the colonies were initially looking creamy white. But some gradually turned dark at the middle indicating little microsclerotia production, while others remain creamy, all colonies were densely packed on to the media with no aerial mycelium. Abundant hyaline and oval conidia were produced by all colonies in both D and ND isolates (Figure 3a and b).

At 28℃ treatment, the D-group recorded significantly higher growth (the highest been HB42 = 48.27mm) compared to ND-strains (the highest been D08165 = 42.33 mm) as shown in (Table 3.0 above; Figure 4b). There is no significant variation in the mean growth responses within the D-strains, but there is significant variation in the mean growth responses among the ND-strains at 28℃ (intragroup variation), for example, D08165 (42.33 mm) is significantly higher than XJ-14 (37.13 mm) and XJ-14 (37.13 mm) is significantly higher than D08047 (34.93 mm) as shown in (Table 3.0; Figure 3.b; Figure 5 b). The entire D-group also showed significantly higher thermal tolerance (higher growth response) at 28℃ level compared to ND-group [Table 3.0; Figure 4 b; and Figure 5b].

While at 30℃ maximum mean growth among the D-group was recorded by HB-19 (40.333mm) while XJ-36 (36.93mm) recorded the lowest mean growth, while among the ND-strains XJ-02 (26.93 mm) recorded the highest mean growth and XJ-63 (22.07 mm) has the lowest mean growth (Table 3.0; Figure 4 c; and Figure 5 c). The entire D-strains showed significantly higher mean growth response (or thermal tolerance) at 30℃ condition compared to the ND-group [Figure 4 c; and Figure 5c; Table 3.0].

Whereas at 32℃ conditions, the highest mean growth was recorded by XJ28 (28.4mm), while HB-10 (24.73mm) recorded the lowest mean growth among the D-strains (Table 3.0; Figure 4d; Figure 5d). Whereas among the ND-strains, isolates XJ-02 (18.27mm) has the highest mean growth response while D08047 (14.60mm) recorded the lowest mean growth response [Table 3.0; Figure 4d; Figure 5d]. In general, the defoliating pathotype strains displayed significantly higher mean growth (or thermal tolerance) at 15^{oC}, 28^{oC}, 30℃, and 32℃ conditions compared to the ND-strains [Table 3.0; Figure 4; Figure 5] andresult of this study aggreed with the Xu et al. (2012).

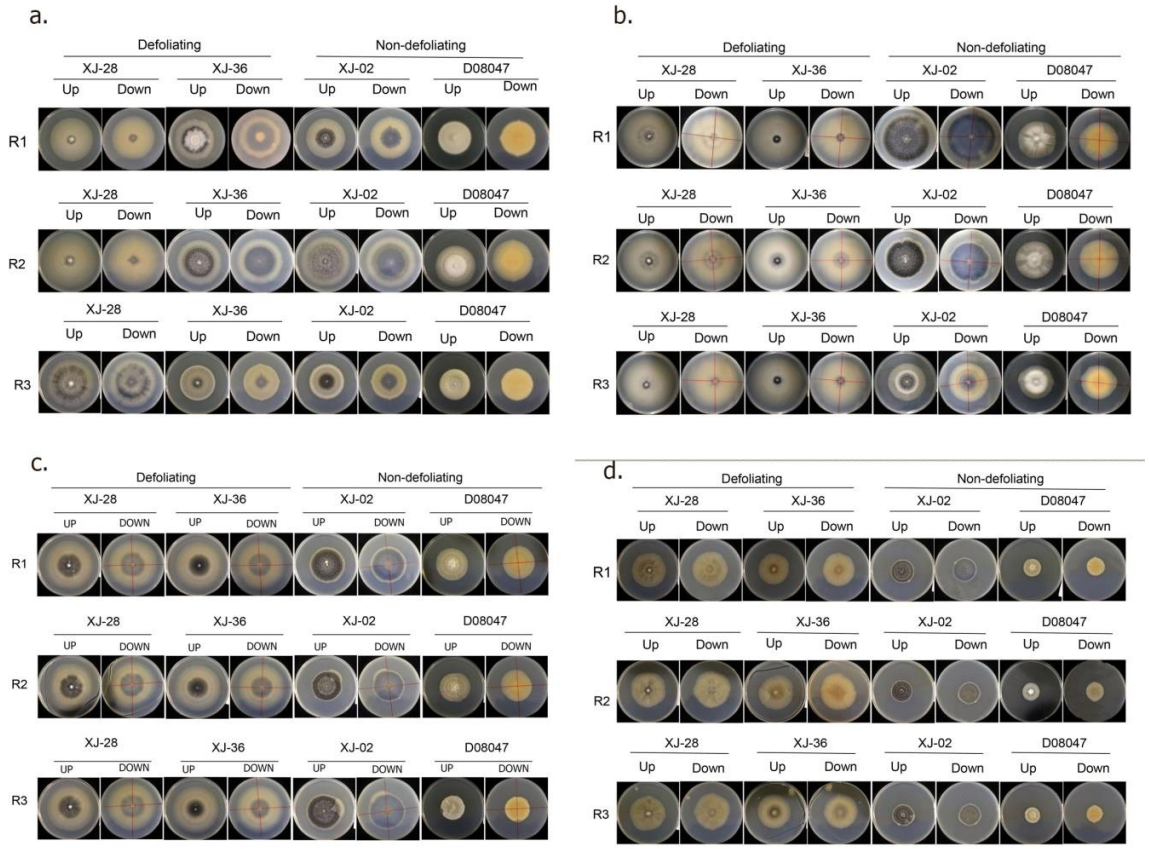

FIGURE 4. GROWTH RESPONSE PHENOTYPES OF D AND ND OF V. DAHLIAE TEST ISOLATES AT 15℃, 28℃, 30℃, AND 32℃. WHERE A = PHENOTYPES AT 15℃; B = PHENOTYPES AT 28℃; AND C = PHENOTYPES AT 30℃; AND

D = PHENOTYPES AT 32°C; R1, REPRESENTS REPLICATION 1; R2 = REPLICATION 2 & R3 = REPLICATION 3 IN EACH OF THE THREE CASES. UP = FACE SIDE AND DOWN = BOTTOM SIDE OF THE CULTURE PLATE/PHENOTYPE.

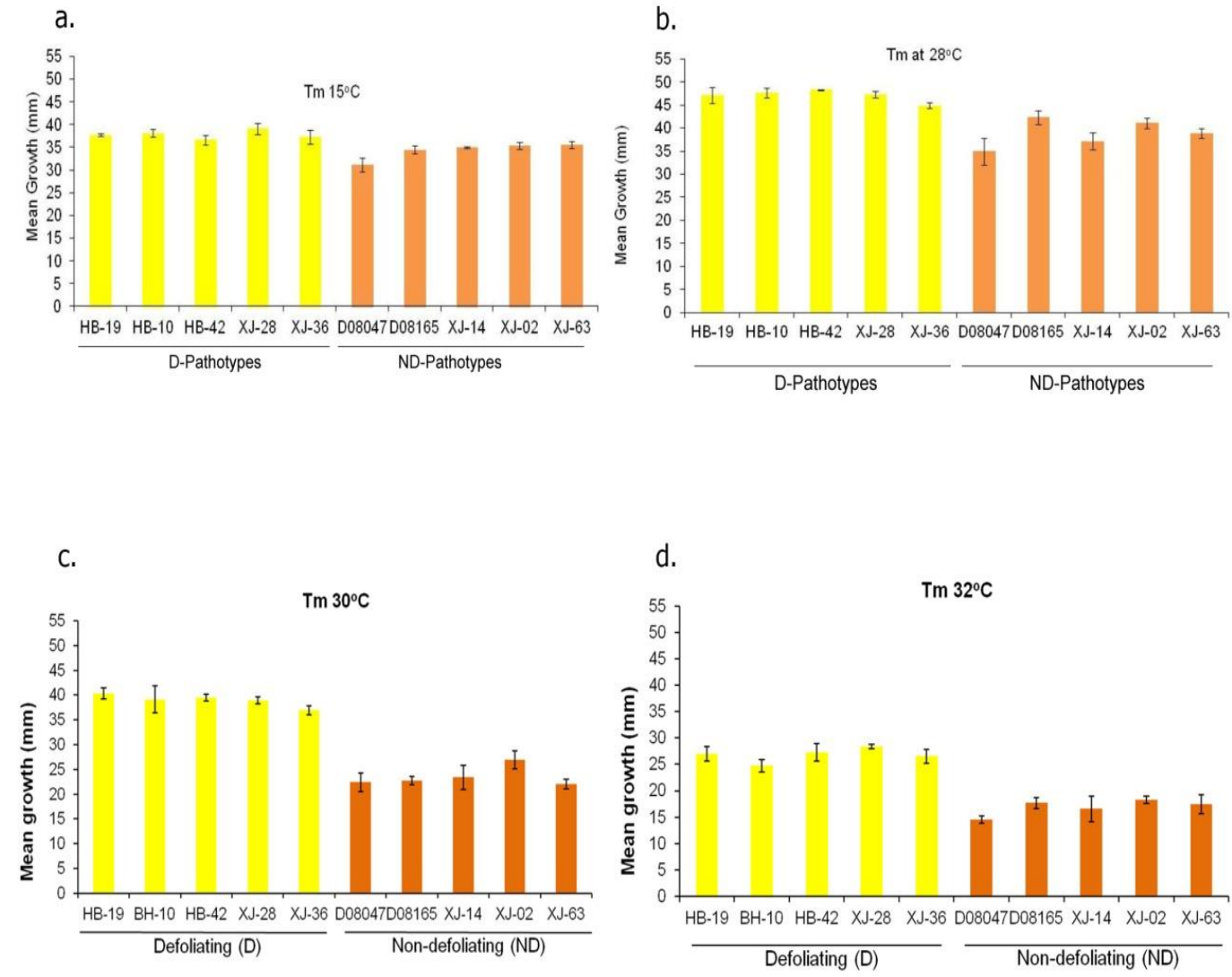

Figure 5. Mean mycelial growth of the test isolates at Tm (15°C, 28°C, 30°C, and 32°C). Whereas Tm = temperature, Y-axis represents the growth response of V. dahliae, and X-axis represents isolates. a shows the response at 15°C for the D and ND, b = shows the response at 28°C for the D and ND; c = displays growth response for the D and ND at 30°C, whereas d = shows the response at 32°C for both D and ND.

Melanization varied between D and ND, and from one temperature to another:

The appearance (phenotypes) of V. dahliae cultures on PDA or other artificial media differ among isolates belonging to the same species (Jabnoun-Khiareddine, 2006). The abundance or not of microsclerotia and its distribution in V. dahliae culture growing on PDA can give many differences in the morphology of strains/isolates. Media have also been used to differentiate one Verticillium species from another, e.g., oat medium, which promotes faster growth of V. albo-atrum isolates compared to strains from V. dahliae (Jabnoun-Khiareddine, 2004).

The amount of pigmentation varies between the D-pathotypes and ND-pathotypes and between one temperature and another. In general, the D-pathotypes showed significantly more Melanization at 15°C, 30°C, as well as 32°C (Figure 6 a), compared to isolate from the ND-pathotypes (Figure 6 b). In contrast, isolates from the ND-pathotypes are generally more melanized relative to isolates from the D-pathotypes at 25°C. D-pathotypes isolates produced more of dark grey pigmentation (Figure 6 a), but ND-pathotypes produced dark pigmentation

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(Figure 6b). There is no aerial mycelial growth observed from both groups at the entire Tm tested (Figure 6a & b).

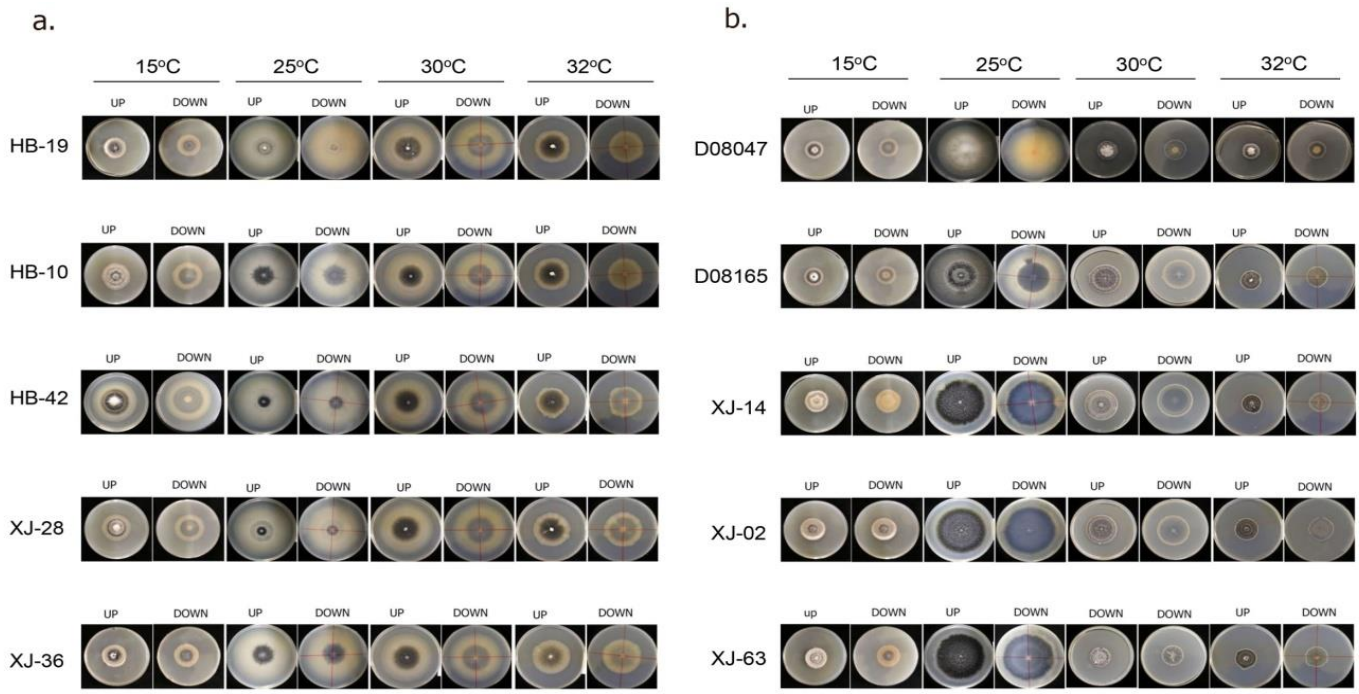

Figure 6. Test isolates showing differences in melanin at 15°C, 25°C, 30°C, and 32°C. a. shows melanization in the mycelium of the D-pathotypes at different Tm level; b. shows melanization in the mycelium of the ND-pathotypes at various Tm. Up = face or surface picture; Down = bottom picture of the phenotype

DISCUSSION

Various studies were conducted across the globe attempted at assessing the variation between D-strains and ND-strains in various ways, and this study aims to explore the variation in mycelial growth, conidial size, and melanization of the two pathotypes in response to Tm treatments. We observed that isolates from D-pathotypes differed significantly from their ND counterpart in their response to temperature for in vitro growth at a certain temperature. The entire D-strains showed significantly higher growth response (i.e higher temperature tolerance) at 15°C, 28°C, 30°C and 32°C conditions compared to the ND-pathotypes (Table 2.0 & Table 3.0; Figure 4 & Figure 5). The results in this study which shows D-strain to adapt to the two opposites extreme temperatures (15°C; 28°C; 30°C, & 32°C) are in agreement with earlier findings reported in Shi et al. (1993), Bejarano-Alcázar et al. (1996), Korolev et al. (2008), and Xu, et al. (2012). Xu, et al. (2012) discovered that the D-pathotype isolates could well adapt to high temperature and heavily infect cotton under 25–30°C, and these features might be responsible for the rapid spread of this pathotype in central China. But our result shows, that the in-vitro growth response of the D- pathotypes are not significantly better than that of the ND-pathotypes (i.e., are both better adapted to 25°C- 20°C) which is not in agreement with findings of Bejarano-Acázar, J. et al. (1996), but it's observed that D-pathotypes can as well significantly adapted to the 28°C condition better than the ND-pathotypes (Table 2.0; Table 3.0; Figure 4, & Figure 5). This supports the findings in Xu, et al. (2012), who reported that "D-pathotype isolates adapted better than the ND-pathotype isolates to 30°C for conidial germination and mycelial growth, although the isolates of the two pathotypes had the same optimum temperature of 25°C".

Xinjiang is a landlocked region in western China, surrounded by high mountains with hot and humid during summer, the muggier period of the year lasting for about six months from March 31 to October 27, during which the comport levels is muggy, oppressive or to say miserable with at least 25% of the time muggy. July 9 has the muggiest conditions 100% of the time (weatherspark.com on 2020/03/30), while Hubei is located in the middle reaches of the Yangtze River, where it's hot and humid during summer (Xu, et al. 2012). Our findings on this study suggest the above weather conditions might play a significant role in favoring the D-pathotype adaptation to the niche faster than the ND-pathotypes, which confirmed the earlier studies reported by Xu, et al. (2012).

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Verticillium dahliae produces resting structures called microsclerotia (MS) that can last for many years, and this constitutes the main potential infective propagules of the pathogen in seeds or soils. These MS are produced in senescent or dead tissues of the affected plants (Lopez-Escudero & Mercado-Blanco. 2011). Microsclerotia and melanized particles in fungal inter-hyphal spaces confer resistance to extreme temperature, UV irradiation, enzymatic lysis, and fungicidal activities of their host plant (Gordee & Porter, 1961; Griffiths, 1970; Gessler et al., 2014). Wilhelm (1955) has reported broad differences in MS size and shape, and V. dahliae isolates form smaller oval to elongated MS, which are different from the hyaline mycelium and conidiophores due to melanization.

Differences among Verticillium species regarding the amount and morphology of the MS produced on potato dextrose agar (PDA) are pronounced. In this study melanization of the five isolates from D-pathotypes on PDA were significantly higher compared to melanization in the five isolates from the ND-pathotypes at 15℃, 30℃ and 32℃, but ND-pathotypes were more melanized at 25℃ (Figure 5).

Dark pigmentation in the D-strains at a lower temperature (15℃) could be an adaptation to cold environments as reported by (Kalmus, H. 1941) that pigmentation in an organism can be induced by as it attempts to adapt to cold. In contrast, darker-colored species absorb more solar radiation and benefit from higher heating rates, leading to an increase in their overall body temperatures. But in contrast, light-colored species in warm environments should benefit from enhanced reflection of radiation and hence a reduced risk of over-heating. While melanization at higher temperature might be due to exchange of carbon to biomass production for the thermal regulation as highlighted in (Hottiger, et al. 1987), and this could support the heavy melanization in the ND-strains at 25℃ which might be due to low nutrient or due to light.

Results from our on phenotypic differences among melanised isolates from D and ND groups of V. dahliae agreed with earlier findings of (Goud et al. 2003). Goud et al. (2003), López-Escudero, and Blanco-López (2005) used melanized microsclerotia as a morphological criterion for establishing a suitable distinction among species and sub-specific groups, which may display differential pathogenicity or virulence, and we know microsclerotia are positively correlated with melanization in V. dahliae.

Many studies have reported that morphology of MS produced by V. dahlia in different culture media might be correlated with the virulence of the isolate and we intend to test this hypothesis in the context of melanization/pigmentation in the next experiment (in Chapter four; Effect of Tm treatment on Pathogenicity of D and ND-pathotypes). The result of this study highlighted that temperature could affect conidial properties, such as conidial sizes, melanization, stress tolerance, and mycotoxin, which supports the work of Hagiwara, et al. (2016). Increase in conidial sizes at higher temperature could as well be due to phosphatidic acids, and cell membrane fluids as reported in Ganoderma lucidum (Liu et al., 2017a; 2017)

We noted that our isolates exhibited some level of different melanization in response to temperature stresses. This difference in melanization is empirically acceptable for isolates of identical species, e.g., V. dahlia, possess a genetically diverse background, consequently can exhibit some degree of differences in phenotypes, such as hyphal growth, conidiation, conidial melanization, nutrient preferences, and virulence [Kowalski, et al., 2016; Fuller, et al., 2016], but we tried to give an average picture of each group.

Filamentous fungi actively produce conidia (asexual spores) under appropriate conditions, and these conidia are reproductive structures that play crucial roles in the distribution and survival of the fungi (Hagiwara, et al., 2017).

Fungi protect themselves from such abiotic stresses by accumulating compatible solutes. Mannitol and trehalose are the most prevalent solute sugars that accumulate in conidia (Wyatt, et al., 2013). Mannitol is the most abundant solute in conidia of Aspergillus oryzae and Aspergillus niger, while trehalose is the major compatible solute accumulates in conidia of Aspergillus fumigatus [Sakamoto, 2009; Hagiwara, et al., 2014; 2016]. The absence of compatible solutes in conidia of A. niger and Aspergillus nidulans reduce their ability to heat resistance hence reduced their longevity [Ruijter, et al., 2003; Sakamoto, 2009; Jørgensen, et al., 2011; Hagiwara, et al., 2014; 2016].

Summary, we screened 10 test isolates of V. dahliae from Xinjiang and Hubei provinces (two major cotton-belts in China), for their in vitro growth responses at six Tm treatments conditions. We found diverse reactions to temperature treatments, both within and without strains as well as within and between temperature conditions. This result can offer a good understanding of the epidemiology of thermal tolerance of Verticillium dahliae and its niche expansion in China, especially Xinjiang and Heibei provinces, hence vital tool for appropriate strategies in management of cotton wilt.

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Authors' contributions

Competing interest: The authors of this manuscript declared no competing interest**.**

Data availability

Supporting Information

REFERENCES

1. Ahuja, I., de Vos, R.C., Bones, A.M., and Hall, R.D. (2010). Plant molecular stress responses face climate change. Trends in plant science, 15(12), 664–674. https://doi.org/10.1016/j.tplants.2010.08.002
2. Bejarano-Alcázar, J., Blanco-López, M.A., Melero-Vara, J.M., and Jiménez-Díaz, R.M. (1996). Aetiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. The American Phytopathological Society.Plant Disease, 80(11), 1233-1238. DOI: 10.1094/PD-80-1233.
3. de Jonge, R., van Esse, H.P., Maruthachalam, K., Bolton, M.D., Santhanam, P., Keykha, Saber, M., et al. (2012). Tomato immune receptor Ve1 sthe recognises effector of multiple fungal pathogens uncovered by genome and RNA sequencing. Proceedings National Academy of Science, U S A. 109: 5110–5115.
4. Foden, Wendy., Georgia Mace, Jean-Christophe Vié, Ariadne Angulo, Stuart Butchart, Lyndon DeVantier, Holly Dublin, Alexander Gutsche, Simon Stuart, and Emre Turak. (2008). Species Susceptibility to Climate Change Impacts. in The 2008 Review of the IUCN Red List of Threatened Species, edited by Jean-Christophe Vié, Craig Hilton-Taylor, and Simon N. Stuart. IUCN Gland, Switzerland.
5. Fuller, K.K., Cramer, R.A., Zegans, M.E., Dunlap, J.C., and Loros, J.J. (2016). Aspergillus fumigatus photobiology illuminates the marked heterogeneity between isolates. Molecular Bio 7: e01517–16. 36. https://doi.org/10.1128%2FmBio.01517-16
6. Gessler, N.N., Egorova, A.S., and Belozerskaia, T.A. (2014). Melanin pigments of fungi under extreme environmental conditions (review). Prikladnaia biokhimiia mikrobiologiia, 50(2), 125-134. https://doi.org/10.1016/j.tplants.2010.08.002
7. Gordee, R., and Porter, C. (1961). Structure, Germination, and Physiology of Microsclerotia of Verticillium albo-atrum. Mycologia.53(2), 171-182. https://doi.org/10.2307/3756235.
8. Goud JC, Termorshuizen J, Gams W (2003) Morphology of Verticillium dahliaeand tricorpus on semi-selective media used for the detection of V. dahliae in soil. Mycological Research. 107:822–830. https://doi.org/10.1017/S0953756203008050
9. Griffiths, D. (1970). The fine structure of developing microsclerotia of Verticillium dahliaeArchiv für Mikrobiologie, 74(3), 207-212. https://doi.org/10.1007/BF00408881
10. Hagiwara, D., Sakamoto, K., Abe, K., and Gomi, K. (2016). Signaling pathways for stress responses and adaptation in Aspergillus species: stress biology in the post-genomic era. Bioscience, Biotechnology andBiochem 80: 1667–1680.

11. Hagiwara, D., Suzuki, S., Kamei, K., Gonoi, T., and Kawamoto, S. (2014). The role of AtfA and HOG MAPK pathway in stress tolerance in conidia of Aspergillus fumigatus. Fungal Genetics and Biology.73: 138–149. https://doi.org/10.1016/j.fgb.2014.10.011
12. Hottiger, T., Schmutz, P., and Wiemken, A. (1987). Heat-induced accumulation and futile cycling of trehalose in Saccharomyces cerevisiae. Journal of Bacteriology, 169(12), 5518-5522. https://doi.org/10.1128%2Fjb.169.12.5518-5522.1987
13. Inderbitzin, P., Bostock, R.M., Davis, R.M., Usami, T., Platt, H.W., et al. (2011a). Phylogenetics and taxonomy of the fungal vascular wilt pathogen Verticillium, with the descriptions of five new species. PLoS ONE, 6: e28341. https://doi.org/10.1371/journal.pone.0028341
14. Inderbitzin, P., Davis, R.M., Bostock, R.M., and Subbarao, K.V. (2011b). The Ascomycete Verticillium longisporum is a hybrid and a plant pathogen with an expanded host range. PloS one, 6(3). : e18260. https://doi.org/10.1371/journal.pone.001826
15. Inderbitzin, P., Davis, R.M., Bostock, R.M., and Subbarao, K.V. (2013). Identification and differentiation of Verticillium species and longisporum lineages by simplex and multiplex PCR assays. PloS one,8(6). https://doi.org/10.1371/journal.pone.0065990
16. Jabnoun-Khiareddine, H. (2004). Verticillium wilt of vegetables in Tunisia: Causal agent, pathogenicity, and control measures. M.Sc. Thesis, Plant and Environment Protection of Horticultural High School of Chott Mariem, Tunisia, pp: 87.
17. Jeger, M.J., Hide, G.A., Van den Boogert, P.H.J.F., Termorshuizen, A.J., and Van Baarlen,
18. Chen Jie-Yin, Hong-Li Xiao, Yue-Jing Gui, Dan-Dan Zhang, Lei Li, Yu-Ming Bao, and Xiao-Feng Dai (2016). sCharacterisation of the Verticillium dahliaeExoproteome Involves in Pathogenicity from Cotton-Containing Medium. Frontiers in Microbiology.7:1709.
19. Jørgensen, T.R., Park, J., Arentshorst, M., van Welzen, A.M., Lamers, G., Vankuyk, P.A., et al. (2011). The molecular and genetic basis of conidial pigmentation in Aspergillus niger. Fungal Genetics and Biol 48: 544– 553.
20. Jyoti, S., Singh, D.P. (2016). Production of Secondary Metabolites from Two Penicillium Strains Adapted to Different Temperature Conditions: A Study on Differential Response of Fungal Strains to Temperature Stress. Cell Mol Biol 62: 136. Available from: https://www.researchgate.net/publication/312044694_Production_of_Secondary_Metabolites_from_Two_Penicillium_Strains_Adapted_to_Different_Temperature_Conditions_A_Study_on_Differential Response_of_Fungal_Strains_to_Temperature_Stress [accessed May 20 2020].
21. Kalmus, H. (1942). The survival and change of weight on sugarwater mixtures of Drosophila mutants and species of different body colour. Journal of Genetics, 44, 194-203. https://www.ias.ac.in/article/fulltext/jgen/044/02-03/0194-0203 Access on 08/07/2024
22. Klosterman, S.J., Subbarao, K.V., Kang, S., et al. (2011). Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. PLoS Pathogens. 7(7):e1002137. DOI:10.1371/journal.ppat.1002137
23. Korolev, N., Pérez-Artés, E., Mercado-Blanco, J. et al.(2008). Vegetative compatibility of cotton-defoliating Verticillium dahliae in Israel and its pathogenicity to various crop plants. European Journal of Plant Pathology. 122, 603.
24. Kowalski CH, Beattie SR, Fuller KK, McGurk EA, Tang Y, Hohl TMObar JJ, Cramer RA.2016.Heterogeneity among Isolates Reveals that Fitness in Low Oxygen Correlates with Aspergillus fumigatus Virulence. mBio7:10.1128/mbio.01515-16.https://doi.org/10.1128/mbio.01515-16
25. Milgroom, M.G., del Mar Jimenez-Gasco, M., Concepcíon, O-G., and Jiminéz-Diaz (2013). New insights on the phylogenetic relationships between strains of Verticillium dahliae. In: Koopmann B, Tiedemann Av, editors. 11th International Verticillium Symposium - Göttingen Braunschweig: DPG-Verlag.
26. Liu, Y. N., Lu, X. X., Chen, D., Lu, Y. P., Ren, A., Shi, L., et al. (2017a). Phospholipase D and phosphatidic acid mediate heat stress-induced secondary metabolism in Ganoderma lucidum. Environmental Microbiology. 19, 4657–4669.
27. Liu, Y. N., Zhang, T. J., Lu, X. X., Ma, B. L., Ren, A., Shi, L., et al. (2017b). Membrane fluidity is involved in the regulation of heat stress-induced secondary metabolism in Ganoderma lucidum. Environmental Microbiology. 19, 1653–1668.

28. López-Escudero, F.J.; Mercado-Blanco, J. (2011). Verticillium wilt of olive: A case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil.344, 1–50. https://doi.org/10.1007/s11104-010-0629-2. [Google Scholar] [CrossRef] [Green Version]
29. López-Escudero, F. J., & Blanco-López, M. A. (2005). Isolation and Morphologic Characterization of Microsclerotia of. Biotechnology, 4(4), 296-304.
30. Pérez-Artés, E., Garcia-Pedrajas, M., Bejarano-Alcazar, D., and Jime-nez-Díaz, R. M. (2000). Differentiation of cotton-defoliating and nondefoliating pathotypes of Verticillium dahliaeby RAPD and specific PCR analyses. European Journal of Plant Pathology. 106, 507–517.
31. Requena, J.M. (2012). "The stressful life of pathogenic Leishmaniaspecies," in Stress Response in Microbiology. In J.M. Requena, Ed.Caister Academic Press.
32. Ruijter GJ, Bax M, Patel H, Flitter SJ, van de Vondervoort PJ, de Vries RP, et al. (2003). Mannitol is required for stress tolerance in Aspergillus niger Eukaryot Cell, 2: 690–698.
33. Sakamoto K, Iwashita K, Yamada O, Kobayashi K, Mizuno A, Akita O, et al. (2009). Aspergillus oryzae atfA controls conidial germination and stress tolerance. Fungal Genetics and Biol 46(12): 887–897. DOI: 10.1016/j.fgb.2009.09.004
34. Short, D. P. G., Gurung, S., Maruthachalam, K., Atallah, Z. K., and Subbarao, K. (2014a). Verticillium dahliae race 2-specific PCR reveals a high frequency of race 2 strains in commercial spinach seed lots and delineates race structure. Phytopathology. 104:779-785.
35. Short, D.P., Gurung, S., Hu, X., Inderbitzin, P., and Subbarao, K.V. (2014b). Maintenance of sex-related genes and the co-occurrence of both mating types in Verticillium dahliae. PloS One. 9(11):e112145. DOI: 10.1371/journal.pone.0112145.
36. Short, D.P.G., Kerry, O., and Geiser, D.M. (2014c). Clonality, recombination, and hybridization in the plumbing-inhabiting human pathogen Fusarium keratoplasticum inferred from multilocus sequence typing. BMC Evolutionary Biol 14: 91.
37. USAMI, T., ABIKO, M., SHISHIDO, M. et al.(2002). Specific Detection of Tomato Pathotype of Verticillium dahliae by PCR Assays. Journal of General Plant Pathol 68, 134–140. https://doi.org/10.1007/PL00013066
38. Walther, G., Post, E., Convey, P. Mendel, A., Parmensan, C., Beebee, T.J.C., Fromentin, J., Hoegh-Guldberg, O., and Barlein, F. (2002). Ecological responses to recent climate change. Nature,416, 389–395.
39. Wilhelm, S. (1955). Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology, 45, 180-181.
40. Wyatt, T.T., Wo¨sten, H.A., and Dijksterhuis, J. (2013). Fungal spores for dispersion in space and time. Advances in Applied Microbiology. 85: 43–91. https://doi.org/10.1016/B978-0-12-407672-3.00002-2 PMID: 23942148
41. Xu, F., Yang, L., Zhang, J., Guo, X., Zhang, X., and Li, G. (2012). Effect of temperature on conidial germination, mycelial growth, and aggressiveness of the defoliating and nondefoliating pathotypes of Verticillium dahliae from cotton in China. Phytoparasitica, 40, 319-327.
42. Zhang DD, Wang XY, Chen JY, Kong ZQ, Gui YJ, Li NY, Bao YM, Dai XF. (2016). Identification and characterization of a pathogenicity-related gene VdCYP1 from Verticillium dahliae. Nature Scientific Report. 22; 6: 27979. doi: 10.1038/srep27979. PMID: 27329129; PMCID: PMC4916405.