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Trade-Offs between Growth Rate, Sporulation and Pathogenicity in *Verticillium dahliae*

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Abstract

Verticillium dahliae is a soil-borne phytopathogenic fungus that causes vascular wilt diseases in cotton (*Gossypium hirsutum* L.). We have illustrated the correlations between growth rate, sporulation and pathogenicity in *V. dahliae* mutants. A total of 196 *V. dahliae* mutants generated by *Agrobacterium tumefaciens*-mediated transformation, was used throughout this study. Compared with a control strain, the mutants could be significantly grouped into three types by differentiation with to that of control strain. The results indicated that linear regression analysis of the relationship was performed and the gradient and intercept were shown for growth rate and disease index. The correlation coefficient was observed if the infection is high. When their correlation of the growth rate is high, the growth rate and sporulation quantity of mutants had significantly a power correlation. This study, which may play an important role in the population structure of the pathogen, highlights fundamental biological differences between the mutants involving different infection strategies.

Keywords: *Verticillium dahlia*, growth rate, sporulation, pathogenicity

1. Introduction

Verticillium dahliae Kleb is a soilborne fungal pathogen that belongs to Ascomycetes and causes vascular wilt in a broad range of dicotyledonous host plants (Pegg & Brady, 2002; Shuai et al., 2014; Xiao & Subbarao, 1998). A list of infected hosts by the pathogen is continually expanding as disease outbreaks on new hosts which are identified (Bishop & Cooper, 1983; Garas, Wilhem, & Sagen, 1986; Wilhelm, 1955). Serious outbreaks due to *V. dahliae* can cause severe yield losses in crops such as cotton, cauliflower and sunflower (Bhat, Smith, Koike, Wu, & Subbarao, 2003; Fradin et al., 2011; Gao et al., 2013). Once the plant is diagnosed with Verticillium wilt since then there is no fungicide that can cure it (Fradin & Thomma, 2006). Three vegetative phases make up *V. dahliae*'s life cycle, i.e., dormant, parasitic and saprophytic and no sexual life cycle is known so far (Klimes & Dobinson, 2006). The broad host range and long-term survival of microsclerotia in soil make this pathogen difficult to manage in agricultural systems. Moreover, *V. dahliae* is known to disperse by the movement of soil, irrigation water and with seed and vegetative propagation (Koike, Subbarao, Davis, Gordon, & Hubbard, 1994). Some clonal lineages have been dispersed across broad geographic distances (Conn, Tenuta, & Lazarovits, 2005) by agriculture. Physical and chemical factors have pronounced effective control on this disease that depend on the understanding of the complex factors governing the epidemiological process of this pathogen such as growth rate and sporulation (on artificial media which are important biological characteristics), pathogenicity and their interactions. Till today, all these aspects are still under study and the pathogen population is not well characterized and little is known about its prevalence in *Verticillium dahliae*. Morphological characteristics of fungal pathogens are studied at both asexual and sexual stages for the identification of these pathogens. In the case of some fungal pathogens, specific conditions are provided for sporulation (production of spores/conidia) and the morphological criteria are determined for appropriate placement in different species, genera, family etc. (Debode et al., 2007; Fradin et al., 2009). In the present study, we are comparing the growth rate, sporulation and pathogenicity of *V. dahliae* and its mutants. Further, we also measure these mutants *in vitro* and examine them for possible correlations.

2. Materials and Methods

2.1 Fungal Isolates and Culture Conditions

A total of 196 mutants were generated from virulent defoliating *Verticillium dahliae* strains in cotton (Figure 1) by *Agrobacterium tumefaciens*-mediated transformation method described by Maruthachalam et al. (2008) and that was followed with some modifications. These mutants were inoculated onto PDA plates and incubated for 7-10 days at 25 °C in the dark until colonies developed. Single-spore isolates of *V. dahliae* were obtained, identified as previously described (Goud, Termorshuizen, & Gams, 2003; Hawksworth & Talboys, 1970) and maintained on PDA medium in tubes at 10 °C for further use. Then these mutants were confirmed by PCR analysis with *hph*-specific primers (data not shown). The isolates were stored in microconidial suspension in 25 % glycerol at -80 °C till they were used.

2.2 Growth Rate and Sporulation

To measure the growth rate and sporulation *in vitro*, we were selected the Czapek Dox and PDA as basic medium (Zhang et al., 2012). The pH of the test media were maintained at 5.5 and which were optimal for the growth and sporulation in majority of fungi. Wild type and its mutants were inoculated and grown on the PDA plates in the dark at 25±1 °C and the growth rates were subsequently calculated based on the colony size. The difference in colony diameter of two adjacent days was determined to represent the colony growth rate. Disks with 6 mm diameter were taken from the edge of colonies and placed onto the center of PDA plates and each mutant was analyzed three times with three replicate. All cultures were incubated at 25±1 °C for 5-7 days in the dark. Each value indicates the mean and standard error.

The spore production quantification of the wild type and mutants were assessed by placing drops of a spore suspension through two ways: Firstly, by using the Petri-dish with a sharp spatula and washing several times with a total volume of 100 mL of sterilized water. The suspension was filtered through a plastic sieve to separate the spores from the mycelium. Secondly, by using the conidial suspension through adding 200 µl of the conidial suspension of 1×10^6 conidia/ml to 50 ml of medium and incubating the cultures at 25 °C, by vigorous shaking 150 rpm for 7 days. To quantify the final conidia concentration of spore, a hemocytometer under a microscope was used. Also here each value indicates the mean and standard error. The linear growth and number of spores produced by each replicate was recorded after 7 days of incubation.

2.3 Pathogenicity Test

Cotton plants (cv. Ji Mian No. 11) were used in the pathogenicity test of wild type and its mutants. A root-dip method was employed for plant inoculation (Gao et al., 2010). Cotton seeds were sterilized on their surface with 70% ethanol for 3-5 min, then with 2% sodium hypochlorite solution (v/v) for 15 min, and then washed three times under sterile water and dried between filter paper before sowing. Seedlings were grown in pots containing disinfested soil and compost mixture (1:1, v/v) under glass house conditions till the two true-leaf development stages. The roots of three weeks age plants were trimmed just before inoculation (Joaquim & Rowe, 1991). Three pots were used for each isolate and every pot contained 5 plants (a total of 15 plants per isolate). Inoculums were harvested from 1-week old liquid cultures on PDB (Potato Dextrose Broth), filtrated using four layers of gauze, and washed with sterilized water twice.

The pathogenicity tests were performed by dipping the plant roots into a conidial suspension 1×10^6 spores/ml which was determined by a hemacytometer under a microscope (Olympus BX51, Japan). Pots were fertilized with the recommended dose of organic and inorganic fertilizers and watered regularly. Fifteen un-inoculated seedlings were dipped in sterile distilled water as control strain. Disease symptoms appeared after 7 days, disease severity was observed and recorded as described by Hu et al. (2013). The experiment was repeated once.

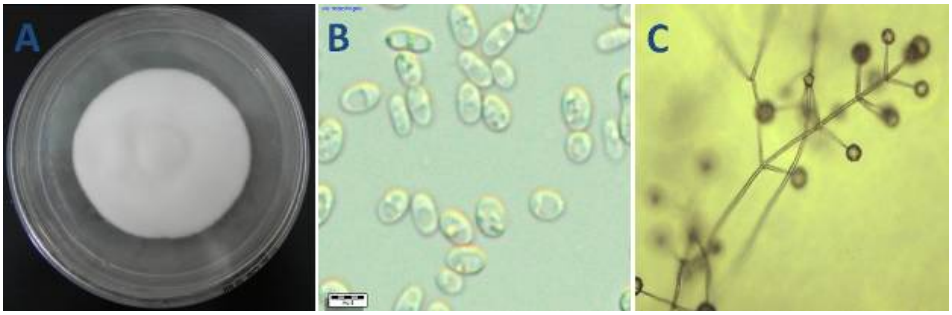

Figure 1. (A) front view of colony morphology on PDA for Wild type; (B) a single spore produced by wild type; (C) branched conidiophores, which form whorls capped with flask-shaped and pointed phialides carrying terminal

2.4 Data Analysis

All experiments were conducted in three replications and repeated 3 times, and the SPSS software was used to analyze the data (Standard Version 16.0, SPSS Inc., Shanghai, China). All of the values are expressed as the mean ± the standard error (SE). The significance of differences was determined by one-way analysis of variance (ANOVA) with Student-Newman-Keuls (S-N-K) and the differences with P values of < 0.05 were considered statistically significant.

3. Results

3.1 Growth Rate

The growth rate of colonies was compared on PDA medium and recorded the highest mycelia diameters after 7 days.In present study the results were subjected to analyze of variance tests at $P < 0.05$ to compare the growth rate between the wild type and its mutant. We observed that they were significantly divided into three groups. Based on their colony growth rate, 35.2% of mutants was significantly reduced the growth rate whereas, about Also 34.7% of mutants was increased their growth rate. No significant difference was found among 30.1% because they had similar growth with the wild type (Figure 2A).

3.2 Sporulation Quantity

In this trail an initial spore concentration of 1×10^6 conidia ml^{-1} was cultured on the PDA medium. Figure 2B showed that, the sporulation quantities of wild strain and its mutants were determined. The sporulation rate was significantly influenced compared with the wild type. The sporulation quantity of 42.9% mutants was much higher than that the wild type, whereas the 30.1% of mutants was recorded lower than that of the wild type. And 27% had similar sporulation quantity to that of wild type strain.

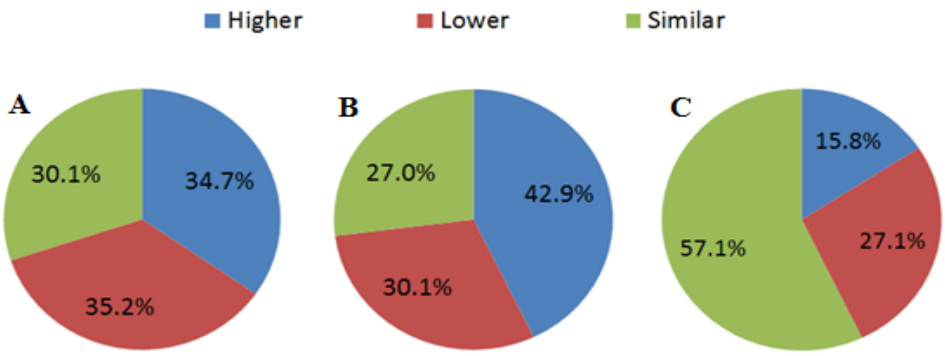

Figure 2. Comparison between the wild type strain as a control and its mutants, the mutants could be significantly grouped into three types by differentiation with to that of control strain. (A) Growth rate. (B) Sporulation quantity. (C) Pathogenicity test

3.3 Pathogenicity Test

Pathogenicity test was performed using the conidia from wild-type strain with cotton cultivar Ji Mian NO11. Infections were performed independently for a period of up to 6wpi to examine the pathogenicity of different genotypes. Symptoms could be observed obviously, including leaf chlorosis and stunting in plants that infected with the wild type and its mutants at 21 dpi. Based on the disease severity, the 196 mutants were significantly divided into three groups which were compared with the wild type. We found out that 15.8% and 27.1% of mutants were higher and lower than that the wild type in pathogenicity respectively. Meanwhile 57.1% of mutants was similar to that of the wild type in pathogenicity (Figure 2C). Table 1 showed that, Comparison between wild type and 25 mutants randomly selected from our experiment results which were measured that the growth rate and sporulation *in vitro* and the pathogenicity *in vivo*.

3.4 The Correlations between Growth Rate, Sporulation and Pathogenicity

In regression analysis, A highly significant correlations were found between the growth rate on the fresh PDA medium of each mutant and the disease index recorded at 21 dpi ($R^2 = 0.9129$), the gradient was 15.54, and the intercept was 48.114. They all were particularly useful for obtaining a linear relationship and explaining a reasonably high percentage of the variation in colony size indicating that the percentage of the variation in disease symptoms. Based on ($R^2 = 0.8936$), the greatest amount of the sporulation quantity and percentage of the variation in colony size had a significantly power correlations with 2.8532 intercept power and with the gradient of 0.012. Our comparison clearly indicates that the growth rate is the key factor for pathogenicity, especially when sporulation is lower than that of control strain (Figure 3).

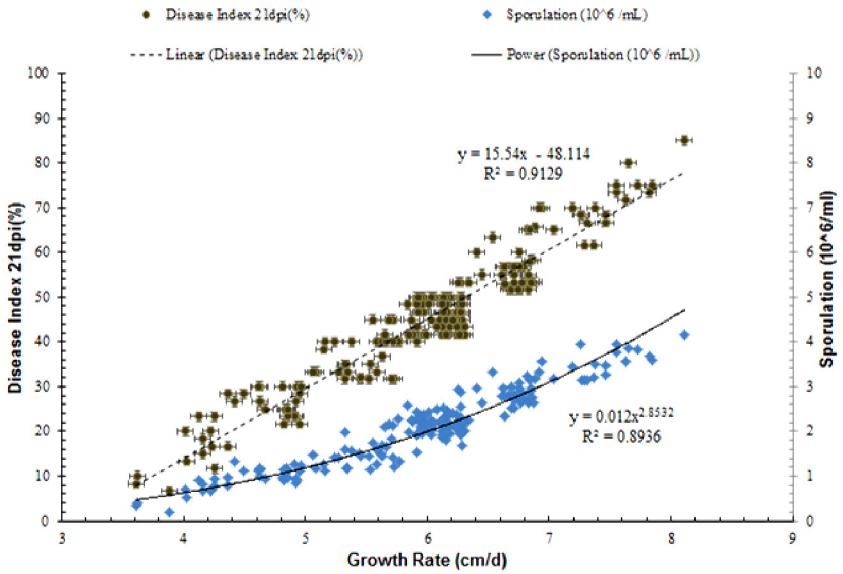

Figure 3. The correlations between growth rate, sporulation and pathogenicity

4. Discussion

Verticillium wilt is typically soil-borne disease. The speed of mycelia growth, the number of spore-bearing and disease index had effects on each other. In our experiment the trade-offs between growth rate, sporulation and pathogenicity *in vitro*/*in vivo* experiments on *V. dahliae* wild type and 196 mutants have been carried out. The study of the growth rate and sporulation *in vitro* are markedly influenced by comparing with the wild type.

Screening of 196 mutants was distinct in providing virtually complete coverage of the value of the molecular mechanisms that involved of *V. dahliae* indirectly by correlations between biological properties. Overall between-the wild type and its mutant trade-offs in performance of multiple-host pathogens are expected to increase under stabilizing selection as the number of hosts and their phylogenetic distance increase in a plant community (Maruthachalam et al., 2008b).

The lifestyle of *V. dahliae* in its center of origin might be shaped by the constraint of short-distance spore dispersal in a high diversity species forest and the fitness costs caused by the need for a multiple-host strategy associated with reduced sporulation.

Such reduced sporulation, however, could be enough for the effective spread of *V. dahliae* in areas where environmental conditions are similar to those expected in its native habitat.

During the study the behavior for *V. dahliae* in terms of growth rate, sporulation and pathogenicity were revealed. In addition, this contrasting behavior for *V. dahliae* underline the presence of probably two infection strategies; the first is based on sporulation for better dispersion of the pathogen and the second is based on growth rate for rapid tissues and vessel colonization. In fact, aggressiveness *in vivo* and *in vitro* tests revealed the best growth rate and the highest disease index. These differences can be explained by differential mutant sensitivity to mycostasis. This has been investigated as a mechanism by which propagules are protected from spontaneous germination in the absence of potentially colonisable substrata (Liu et al., 2013).

In the determination of virulence and sporulation, the sporulation was reduced; however, it could be effective to spread the *V. dahliae* and will not have any difference in its virulence. Plants were subsequently grown under greenhouse conditions for up 16 weeks (isolate comparison study) or 3 to 4 weeks (rapidly study). This assay is preferred because it is rapidly compared with the conventional greenhouse assay in the soil medium (Klosterman & Hayes, 2009). Conventional methods for pathogenicity are laborious and time consuming, and require 3 to 4 weeks for final results.

Our interesting study is for correlating between growth rate, sporulation and pathogenicity in *V. dahliae* mutants. According to our results, 'growth rate' and 'sporulation' strategies are more prevalent in *V. dahliae* in cotton, respectively. Evolutions in the pathogen population structure at one hand and distribution of the disease in the field in time and space on the other hand are required to be kept under investigation for a better disease management.

Table 1. Comparison between the growth rate, sporulation and pathogenicity of wild type and some mutants

Mutant	Disease Index 21 dpi	Growth Rate (cm/d)	Sporulation (10^6 / ml^{-1})
Wild type	56.67	6.96±0.12	2.10±0.10
M10	20.00	7.22±0.05	2.21±0.08
M17	26.67	6.63±0.25	1.80±0.02
M65	25.00	6.67±0.09	2.20±0.04
M49	15.00	5.76±0.16	1.57±0.06
M73	31.67	6.12±0.12	1.15±0.05
M90	46.67	6.45±0.35	1.93±0.04
M94	8.33	7.61±0.63	0.85±0.10
M99	70.00	6.78±0.15	2.20±0.11
M89	41.67	6.88±0.19	1.90±0.08
M128	55.00	7.11±0.19	2.01±0.04
M185	13.33	8.03±0.23	1.63±0.08
M129	16.67	5.46±0.09	1.68±0.04
M77	31.67	7.83±0.12	2.00±0.07
M109	60.00	6.95±0.23	2.08±0.10
M155	11.67	5.66±0.16	1.48±0.08
M147	85.00	7.11±0.18	1.76±0.09
M139	71.67	5.63±0.10	2.05±0.07
M25	48.33	6.56±0.17	1.66±0.10
M67	40.00	6.62±0.05	2.35±0.07
M75	6.67	4.88±0.12	0.90±0.07
M140	21.67	7.82±0.38	2.13±0.06
M190	35.00	7.53±0.10	1.90±0.20

M55	66.67	6.31±0.08	2.10±0.10
M138	45.00	7.60±0.10	2.16±0.10
M47	53.33	6.61±0.06	2.21±0.12

Note. M: Mutant, dpi: days post inoculation. The values in the table above were the average of three replicates for wild-type and its mutants, respectively, and the value following by "±" was standard error. Values were significantly different ($P < 0.05$) in S.N.K test of one-way ANOVA.

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