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A New Detection Method of Wilting Virulence Induced by Phytotoxin from *Verticillium dahliae* on Cotton through Leaf Pricking and Spreading

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Abstract: A new method to measure the virulence of phytotoxin wilting caused by *Verticillium dahliae* through pricking on leaf surface and spreading phytotoxin on pricked area has been developed. First, phytotoxin of *V. dahliae*(*V. d.*-toxin)of V_{991} strain, a severe defoliating virulence strain of *V. dahliae* to cotton, was partially purified with Sephacryl S-200 HR; next, 20~50 pits were pricked with a small needle in 0.5 cm^2 on the surface of cotyledon or euphylla of cotton seedling; then, certain volume of *V. d.*-toxin solution was spread on the 0.5 cm^2 of the leaves; and in 24 h after the treatment, wilting spots were appeared and recorded it. The water and BSA treatments were employed as controls. The virulence of wilting could be divided into five classes from 0 to Ⅳ. Apart from the purified *V. d.*-toxin, the filtrate (liquid culture medium filtered after culturing *V. d.* over 7 days) can be also used for this method. To identify its general use of this method, eight *V. d.* strains were inoculated on leaves of 3 cotton cultivars. These *V. d.* strains included: severe wilting virulence (V_{991}, T_9, V_{56} and V_{146}), weak wilting virulence (V_{110}, V_{246}, V_{250}) and medium wilting virulence ($V_{3\text{-}10\text{-}1}$). By pricking small pits on cotton leaf surface and spreading *V. d.*-toxin onto the pricked area, it was easily to observe the wilting symptom and to evaluate their wilting virulence. With this method, the 8 tested strains were classified into corresponded groups, for instance, V_{120}, V_{991} and T_9 were divided into the severe type of wilting virulence. This grouping result was as same as that formerly conducted through traditional methods (such as soaking seedling method). To determine the optimum cotton growing stage and part of the leaf on cotton plant, both cotyledon and euphylla in seedling and euphylla in adult plant were tested. As the result, this method could be utilized as early as in seedling stage, and cotyledon was better than euphylla since it was easy to prick but not easy to get through. However the extent of wilting varied with the temperature of environment, cotyledon or euphylla, and the concentration of *V. d.*-toxin used. To determine the differential reaction of cotton cultivars to *V. d.*-toxin with this method, four upland cotton cultivars (Ejing-3, B99261, CCRI 12 and Xingluzao-7) were used, and the coherence between *V. d.*-toxin soaking method and our new method was compared. The result proved that both methods got very similar by multiply tests.

From the experiments, this method revealed some advantages, such as swiftness, simplicity, high reliability, minute requirement of *V. d.*-toxin (less than 8 μg), and quickness in symptom development (in 24 h) after treatment etc.. This new method can be used to identify both the resistance of cotton cultivars to *Verticillium dahliae* and the wilting virulence of pathovars of *Verticillium dahliae*.

Key words: cotton; phytotoxin of *Verticillium dahliae*; pricking and spreading method; wilting detection

Introduction

Verticillium dahliae is the pathogen which causes the *Verticillium* disease in China, and the *V. d.*-toxin is the main reason for the plant wil-

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ting. Therefore, using *V. d.* - toxin as a "pathogen" to detect resistance of cotton cultivars has its practical significance[1]. There are mainly two categories of method to identify the resistance of cotton cultivars to *Verticillium* disease, which are the field disease plot methods and the indoor treatment methods. The indoor treatment methods can be further divided into several more detailed methods, including cut or wounded roots dipping in spore liquid, root-dipping, stem injection, plant soaking in *V. d.* -toxin to detect the detachment of cells from root caps and etc.[2-3]. However, each method has some limitations, while it appears some advantages. As a result, it is often that the resistance levels of a same cultivar to *Verticillium* disease were disagreement among different years and with different resistance evaluating methods. To overcome the problem, we proposed many measures in order to eliminate the disagreements and improve the accuracy and repeatability of resistance identification[4-7]. Usually, it is well recognized that use of nonstandard detection method is one of the major obstacles for selection and breeding of highly resistant cotton varieties. In this study, we developed a new method identify the wilting virulence called cotton leaf pricking and spreading method, and used this method to detect the pathogenicities of various *V. dahliae* strains. The result showed that the method seemed as a rapid and feasible way to observe the wilting effect with lower usage of *V. d.* -toxin. Meanwhile we try to reduce the influences of environmental factors on the detection results, and to reflect objectively the wilting virulence from *V. dahliae* and the resistance from cotton varieties.

1 Material and Methods

1.1 *Verticillium dahliae* strains and *V. d.* -toxin

1.1.1 *Verticillium dahliae strains.* In this test, eight *V. d.* strains (severe wilting strains V_{991}, T_9, V_{56}, V146, medium wilting strain $V_{3\text{-}10\text{-}1}$ and weak wilting strains V_{110}, V_{246}, V_{250}) were provided kindly by the Plant Protection Institute of Chinese Academy of Agricultural Sciences. Firstly, the purified toxin of V_{991} was used to preliminary establish the method of pricking and spreading. Then the *V. d.* filtrates from eight other strains of different pathogenicities were used to further verify this method.

1.1.2 *Wilting V. d.-toxin .* The V_{991}, a severe defoliating virulence strain of *Verticillium dahliae* on cotton, was inoculated in 500 mL liquid PDA medium at 25℃, 150 r • min^{-1} and in dark for 15 days. The culture medium was filtrated with neutral filter paper, concentrated to 10 mL by low temperature cryodesiccation, and purified with Sephacryl S-200 HR. The purified wilting *V. d*-toxin protein (*V. d.*-toxin) was diluted by 1, 10, 100, 1000 folds and the protein contents were measured with spectrophotometer PE Lambda Bio20. Then the toxin was further diluted by 1000, 2000, 3000, 5000, 6000 folds for use in wilting test.

1.2 The method of leaf pricking and spreading

1.2.1 *Pricking and spreading of the purified toxin .* Gently pricked 15～20 pits with a small pin on the cotyledon and euphylla of Ejing 3 cotton seedlings at 2-3 euphylla leaf stage, making two wound rounds (each measured about 0.5 cm^2) on the up side of the leaf. The two wound rounds were made on each sides of the main leaf vein. In order to increase efficiency and keep the consistency of the pricking depth, a foam board with 15～20 pins fixed on it was used. According to the leaf thickness, the pins on the foam board were exposed out for 0.3～0.5 mm. When pricking, it would be better to put one hand below the leaves to avoid pierce the leaves through and control the pricking depth. Based on the areas of the pricking, 1～3 μL toxin solution was spread on the pricked area, and the eluent buffer, ddH_2O and the BSA served as controls. The test was repeated for 3 times, and 24 cotton seedlings were treated. The symptoms were recorded every 6 hours after the treatment.

1.2.2 *Pricking and spreading of V_{991} filtrate .* To explore the direct use of the *V. d.* filtrate in

this method, V_{991} culture medium was diluted to ten concentrations ranging from 10 to 100 folds[10]. The hypha and spores were removed from the *V. d.* filtrate. The seedlings of Ejing 3 and sea-island cotton AXMN were pricked and spread with V_{991} filtrate of different concentrations. The test was repeated for five times and ddH_2O was taken as control.

1.2.3 *The treatments of cotton varieties with filtrates from different V. d. biological strains*. To test wilting extents of different strain filtrates on various cotton cultivars, pricking and spreading experiment was carried out on three cultivars of upland cottons, Xinluzao-7, B99261 and CCRI 12, using eight *V. d.* strain filtrates in dilutions of 1, 30, 100 and 300 folds. The cultured media of eight *V. d.* strains were filtrated with neutral filter papers, and centrifuged for 15 min in 14000 $r \cdot min^{-1}$. The ddH_2O served as the control. Eight seedlings of similar growth vigor from each cultivar at growing stage of 3～4 euphylla were chosen for use in the experiment. Three euphylla in each seedling were treated. Two 0.5 cm^2 spots were pricked on the up surface of each leaf. Then one pricked spot was spread with 2 μL ddH_2O (as control) and the other one with filtrate of certain concentration (300, 100, 30 or 1 folds dilution). This test was repeated 3 times. The investigation of wilting symptom has been started at 6 hours after treatment, and in 24 hours the disease symptom was taken as the last record.

1.3 The experiment using method of immersing cotton seedling by filtrates

This experiment was used as a contrastive trial of the experiments with pricking and spreading method. Cotton leaves were soaked in the *V. d.* filtrates of eight different pathogen strains. The cotton cultivars used were Xingluzhao-7, B992614 and CCRI 12. Filtrates in dilutions of 1, 30 and 100 folds for each strain were used, and ddH_2O served as control. Each treatment was repeated for 3 times.

1.4 Classification of wilting and calculation of the disease index

In the picking and spreading method, wilting results were divided into 0～4 classes according to the size of necrosis area. Class 0, no necrosis appeared; class Ⅰ, the area of necrosis was less than 25% of the treated area; class Ⅱ, 25%～50%; class Ⅲ, 50% ～ 75%; class Ⅳ, more than 75%. In the experiment using *V. d.* filtrate soaking method, the classification was as follows: After 72 h treatment, leaves were slightly yellowed (class 0), moderately softened or the area of pathological spots were less than 25% of the whole leave area (class Ⅰ); leaves were severely softened or the area of pathological spots were 25%～50% (class Ⅱ); leaves were wilted 50%～75% (class Ⅲ); the area of pathological spots were more than 75% (class Ⅳ). The same classification methods were used for calculation of the pathological index in field tests.

2 Results and Analysis

2.1 Wilting effects when using the method of pricking and spreading of *V. d.* -toxin

2.1.1 *Wilting property on cotton leaf.* The treatment using pricking and spreading method with V_{991} toxin induced visible disease spots on the leaf surface of cultivar Ejing 3 at 25℃ after 12 hours. The wilting symptom appeared on the treated part of the leaf, and the mesophyll cells were dead. In the severe cases, with only epidermis left, the treated part on the leaf looked transparent. Whereas controls, pricking and spreading with BSA or water, made no necrosis spots and only pricking mark appeared on the leaves. After purified via chromatography, *V. d.*-toxin became much stronger than the crude extracts in inducing wilting. For example, the purified toxin in concentration as low as 20 $\mu g \cdot L^{-1}$ was still more powerful of wilting to Ejing 3 (a sensitive cultivar), whereas the crude *V. d.*-toxin had only weak wilting ability to

Ejing 3 and had no wilting virulence to AXMN (a Sea-island cotton cultivar) when further diluted to 7300 $\mu g \cdot L^{-1}$ (3000 folds). The wilting virulence decreased significantly as the crude extracts of *V. d.*-toxin diluted for 100 times. The tests above have been proven successful by repeating more than 10 times.

2.1.2 *Quantitative analysis of the wilting symptom.* By using the pricking and spreading method, as shown above, the obvious differences in wilting symptom on cotton leaves could be observed when different cotton cultivars were treated, or same cultivar treated with different concentrations of the *V. d.* -toxins. Therefore, with the differences a classification standard for further quantitative analysis was made (Fig. 1). In Fig. 1, the symptom of class IV was caused by V_{991} purified *V. d.*-toxin in concentration of 7.3 $\mu g \cdot L^{-1}$, which seemed much heavier than those by crude filtrates.

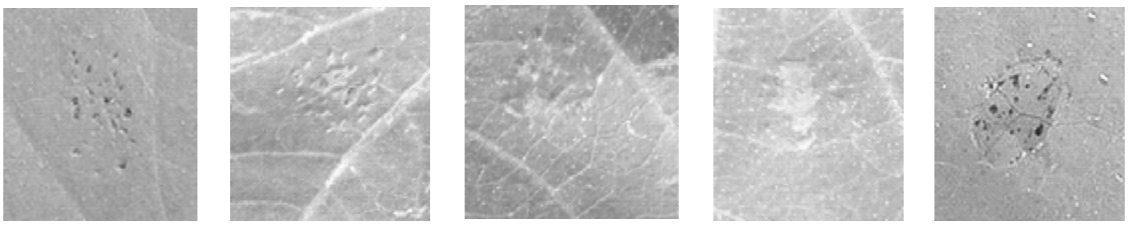
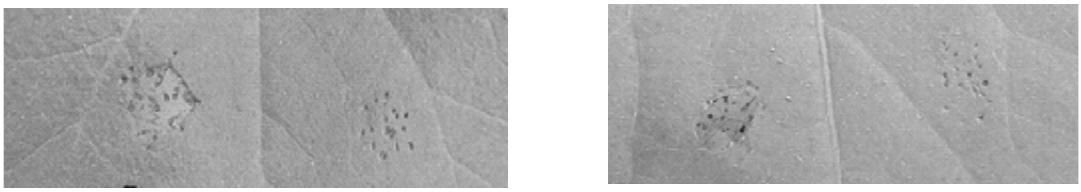


Fig. 1 Wilting classes according to wilting extent by phytotoxin after 24 h when using the pricking leaf and spreading method (class 0～Ⅳ, from left to right)

2.1.3 *Optimum leaf type and cotton growth stage for the treatment.* Cotyledon seemed more suitable than euphylla for this method, since thickly cotyledon had less risk to prick through and was fast in manipulation as well. Some holes by pricking on the euphylla were seen in Fig. 2. The results also showed that this method could be used on cotton from the seedling to tri-euphylla stage. After the tri-euphylla stage, the cotyledon would become shrinking, and the euphylla became thinner leading to high risk for pricking through of the leaves.



The left picture is cotyledon, and the right is euphylla. In the same leaf, the left was treated with phytotoxin and the right was control.

Fig. 2 The wilting symptoms on cotton leaves by pricking and spreading method after 24 h

2.1.4 *The experiment with V_{991} filtrate.* In this test, *V. d.* filtrate could cause the wilting symptom similar as treated with purified *V. d.*-toxin, but the symptom was less severe than that with purified *V. d.*-toxin. When V_{991} filtrate was diluted into 0.0124 $g \cdot L^{-1}$ (60 folds), it was able to cause wilting spots on AXMN and Ejing 3, but no wilting power when the filtrate was diluted to 70 folds.

2.2 Wilting virulence of different strains' filtrates to upland cotton cultivars

2.2.1 *Difference in wilting virulence among V. dahliae strains.* The wilting virulence (indicated by disease index) by this method was generally similar to those by traditional methods. Among the tested strains, V_{120} had a very high protein content in the filtrate (0.1487 $g \cdot L^{-1}$) (Table 1), and accordingly, appeared the most severe wilting virulence, with disease index up to 58.33 on average. $V_{3\text{-}10\text{-}1}$ and T_9 showed severe wilting virulence as well, with disease indexes of 56.25 and 50.00, respectively. However, disease index caused by V_{56} was the lowest (35.42) among the tested strains, dramatically different

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Table 1 Contents of protein in filtrates of *V. dahliae* strains

Strain	OD_{260}	OD_{280}	OD_{230}	Total protein content/(g · L^{-1})
V_{991}	0.0559	0.0363	0.0963	0.0113**
T_9	0.2325	0.1970	0.3329	0.1136
V_{56}	0.2644	0.2287	0.4024	0.1360
V_{120}	0.2909	0.2504	0.4399	0.1478
$V_{8\text{-}10\text{-}1}$	0.1859	0.1608	0.3497	0.0956
V_{110}	0.2256	0.1915	0.3279	0.1107
V_{246}	0.3704	0.3080	0.5058	0.1725*
V_{250}	0.1837	0.1595	0.3285	0.0957

Note: OD values were measured with filtrates diluted by 50 folds.

that evaluated by the traditional method. With the traditional method, V_{56} was evaluated as severe virulence strain. Similar incongruence was occurred with V_{991}, a severe virulence strain evaluated by the traditional method, showing a disease index of 45.83 and protein content of 0.0113 g · L^{-1} in our experiment.

2.2.2 *Difference in wilting resistance to wilting pathogen among cotton cultivars.* The traditional method had proved that the three tested cultivars differed in resistance to *Verticillium* wilt and represented three types of *Verticillium* wilt resistance as high resistance, medium resistance and high sensitivity. The disease indexes of CCRI 12, B99261 and Xinluzao 7 were 18.76, 19.60 and 22.43, respectively, which were evaluated in field disease garden by the Institute of Plant Protection, Chinaese Academy of Agricultural Sciences in 2003.

There were great differences in resistance of same cotton cultivar to different *V. d.*-toxins of *V. dahliae* strains, also to same *V. d.*-toxin by different cotton cultivars (Table 2). For example, the disease index of B99261 was 44.53, the lowest among the three cultivars; whereas that of Xinluzao 7, the highest one, was 49.22. However, when treated with phytotoxin in the suitable dilute fold (30 folds diluted), CCRI 12 showed the highest disease index of 53.12; While treated with phytotoin in a suitable dilute fold (100 folds diluted), Xinluzao 7 showed the weakest resistance with disease index of 65.63; and B99261 showed the medium resistance among the three cultivars, when treated with suitable phytotoxin dilutions of undiluted and 100 folds diluted. This result was consistent with that from field disease garden method by the Institute of Plant Protection, Chinaese Academy of Agricultural Sciences in 2003.

2.3 Comparison of pricking and spreading method with seedling soaking method

2.3.1 *Wilting results using method of soaking cotton leaves with V. d.-toxins.* Filtrates of eight different *V. dahliae* strains were used to treat three cotton cultivars (Xinluzao 7, B99261 and CCRI 12) with diluted into 50 folds. The results also showed the remarkable differences in wilting symptoms among cotton cultivars, *V. dahliae* strains and the interaction between cotton cultivars and *V. dahliae* strains. For example, CCRI 12 could only present wilting symptom by soaking with *V. d.*-toxins of V_{991} and V_{56}; B99261 could present with toxins by V_{991}, V_{120}, T_{91} and V_{246}; and Xinluzao 7 could present the symptom by even more strains. After soaking for 72 hours, results showed that *V. d.*-toxin of V_{991} could cause all three cultivars to be wilted and dried. However, other *V. d.*-toxins of *V. dahliae* could cause only one or two cultivars to be wilted or dried.

2.3.2 *Comparison of pricking and spreading method with seedling soaking method.* The method of pricking and spreading with *V. d.*-toxins could get very similar results as the meth-

od of seedling soaking with *V. d.*-toxins. V_{120}, $V_{3\text{-}10\text{-}1}$ and T_9 were classified as severe wilting

Table 2 Wilting characters differentials by pricking leaf and daubing *V. dahliae* strains' crude phytotoxin on varieties of upland cotton

Treating	Xinluzao 7					Zhongmiansuo 12					B99261					Disease index of *V.* wilt
	CK	300	100	30	1	CK	300	100	30	1	CK	300	100	30	1	
V_{991}	0	0	2	4	0	0	1	0	2	4	0	0	4	1	4	45.83
T_9	0	3	2	3	2	0	3	3	4	2	0	0	0	1	1	50.00
V_{56}	0	2	1	1	0	0	1	0	1	0	0	1	4	2	4	35.42
V_{120}	0	3	4	2	2	0	4	3	4	0	0	0	1	2	3	58.33
$V_{3\text{-}10}$	0	1	4	1	4	0	4	0	4	3	0	2	2	2	0	56.25
V_{110}	0	1	4	3	4	0	1	3	1	0	0	0	1	0	0	37.50
V_{246}	0	0	1	0	1	0	1	4	1	4	0	1	4	1	4	45.83
V_{250}	0	2	3	3	0	0	1	1	0	0	0	3	4	1	4	45.83
Disease Index	0	37.50	65.63	53.13	40.63	0	50.00	43.75	53.12	40.63	0	21.88	62.50	31.25	62.50	
Average	0		49.22			0		46.88			0		44.53			-

Note: Average disease index of each *V. dahliae* strain = sum of data of each classes (300～1 fold)/ (12×4)×100; average disease index of each cotton cultivar = sum of data of each cotton cultivar(V_{991}～V_{250})/(8×4)×100.

virulence by both methods. But there was an exception with V_{991} (as a positive control), which showed a lower wilting virulence (45.83 in disease index) than the that by the seedling soaking method. The reason was probably that the V_{991} had lower toxin content, as it was cultured for only 7 days, less than other strains. However, in the complement experiment with V_{991} condensed toxin solution of 0.1 mg • L^{-1}, the indexes increased up to 69.15, 66.21 and 63.56 on cultivar Xinluzao 7, B99261 and CCRI 12, respectively, which were much similar with results by the seedling soaking method.

3 Discussion

By repeated experiments, it showed that the new method is better to be used with the cotyledon from very young seedling. With properly pricking of the pits on leaves, and suitable phytotoxin or filtrate dilution folds, this method can obtain good identification results in agreement with the traditional method. Furthermore, the wilting symptoms appeared rapidly after treated, making the method suitable for the use in high-through screening. Therefore, this method is valuable to evaluate the wilting virulence of *V. d.*-toxins or filtrates from *V. dahliae* strains, and to identify the *V. dahliae* resistance of cotton cultivars. There is still no determined conclusion whether *Verticillium dahliae* has physiologic races. The reasons include lack of a standard method multiple influence factors and difficulty in qualitative and quantitative analysis of the pathogen. As this new method with much less intermediate steps exclude many factors resulting in the experimental error, it may possibly be used as an assisted method to classify the physiologic types and even the physiologic races of *Verticillium dahliae*.

The time of necrotic lesion appearance after spreading the *V. d.*-toxin, lowest quantity limit of the wilting pathogen to induced wilting symptom and other indexes are closely related to resistance ability of cotton cultivars. The sea-island cotton cultivar used in this study has higher resistance than the upland cotton cultivars, and therefore, the wilting symptoms appeared later on sea-island cotton than upland cotton varieties. This result is consistent with the results from the traditional detection method, which also suggests that the new method can be used in the resistance identification of cotton varieties to

cotton *Verticillium* wilt. Apart from cotton varieties, the speed of symptom appearance after treatment is also related to temperature of treatments. For example, when it is treated at noon, the lesion will appear 6 to 12 hours earlier. In addition, if the treatment and the control are on the same leaf, the control could also appear lesions, which may be caused by the contamination of the toxin to the control area when the leaf is too small. Therefore, it is not suggested to set more than one treatments (apart from control) on a single leaf. As the significant interaction of *V. dahliae* strains × cotton cultivar exists regarding the cotton resistance to the pathogen, therefore, a carefully selected set of multi *V. dahliae* strains is suggested to be used in the evaluation of cotton resistance to *Verticillium* wilt.

In order to verify the application scope, the same experiments were done on tobacco and *Arabidopsis* (data not show). The results indicated that this method is also feasible to be used in these. When tobacco leaves were treated with V_{991} filtrates of 30 and 60 folds dilution, the obvious necrotic lesions appeared at 24 hours after the treatments, while *Arabidopsis* seems not so sensitive to V_{991} filtrate, and with only yellowish leaves at 24 hours after the treatment.

In a word, both methods can be applied to the wilting experiments in the seedling stage of cotton. In contrast to the soaking method, the new method has many advantages, such as symptom appearance in short time, little dosage of *V. d.*-toxin requirement and easiness and accuracy in quantification. This method can be used in identification of *V. d.*-toxin virulence and in screening of the cotton cultivars resistant to *V. dahliae*.

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