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UTILIZATION OF VD TOXIN FOR RAPID SCREENING OF COTTON GERMPLASM AGAINST *VERTICILLIUM DAHLIAE*

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Abstract

The degree of virulence of different isolates of *Verticillium dahliae* (V08sn-1, Anyang and V07df2) was evaluated using pathogen and pathogen free approaches on upland cotton. Direct use of pathogen in the soil classified isolates into highly virulent (V08sn-1), moderate virulent (Anyang) and less virulent (V07df2) with disease index of 65, 40 and 27 on the basis of leaf necrosis and vascular browning. For pathogen free approach, toxins from these isolates were prepared and their protein contents were quantified. Results revealed highest level of soluble protein in V08sn-1 (78 mg/L) followed by Anyang (55 mg/L) and V07df2 (43 mg/L). Similarly, addition of toxin (10 µg/mL) on germinating cotton seeds inhibited radical length in order of V08sn-1(62 %) > Anyang (33%) > V07df2 (17%). Besides, the addition of same quantity of toxin on detached cotton leaves produced marked symptoms of chlorosis/necrosis which was more severe in V08sn-1 and followed by Anyang and V07df2. Moreover, dipping of leaf petiole in Vd toxin of Anyang isolate resulted in leaf wilting in contrast to the leaf dipped in equivalent amount of glucose solution (55 mg/L) which demonstrated that elicitor component of Vd toxin is protein in nature. The results of Vd toxin experiment were consistent with the soil inoculation experiment using *V. dahliae* but the onset of diseased symptoms was quicker in former than later. These findings suggest that utilization of Vd toxin can be an environmental friendly and robust approach for plant breeders to accelerate the process of breeding new resistant lines.

Introduction

Cotton is the world's leading textile fiber and oilseed crop and has a great impact for foil energy and bioengergy production (Zhang *et al*., 2007). Unfortunately, the average annual production loss of cotton is magnificent since last decades in China and the major reason behind is a 'vascular wilt'. The disease is caused by a soil born fungal pathogen *Verticillium dahliae*, which colonizes the vascular tissue 'xylem' as biotroph during the initial phases of infection and causes severe damage in subsequent necrotrophic phase, hence regarded as hemibiotroph (Klosterman *et al*., 2011). Fungal colonization inside the xylem results in vessel occlusion which hinder the transportation of water and nutrients to the aerial parts of the plant resulting in plant wilting, known as *Verticillium* wilt (VW). The pathogen forms microsclerotia as surviving structures that can persist in the soil up to many years (Fradin & Thomma, 2006). Root exudates of the sensitive varieties trigger the growth of fungal hyphae in root zone, followed by its penetration to epidermal cells, root cortex and finally invades the vascular tissue xylem (Huisman, 1982). Control of the *V. dahliae* is difficult, because of the wide host range and extreme variability of pathogenicity. Most of the currently available fungicides are ineffective, once the plants are infected with *V. dahliae* (Fradin & Thomma, 2006) and their continuous use will not only result in resistant pathotypes but will also ascend the environmental pollution. Many other stratigies have been tried by researchers during different time zones to control this devasting disease. For instance, treatment of cotton seeds with known strains of *Pseudomonas* spp. and *Serratia plymuthicaca* (Erdogan & Benlioglu, 2010) and with *Trichoderma virens* (Hanson, 2000) improved the plant resistance against *Verticillium* wilt, while Huang *et al*., (2006) observed decline in yield losses due to *V. dahliae* infestation by using various organic materials. However, none of these approaches could recover the desired targets of crop yield.

Varietal resistance offers the most effective and economical means of reducing the disease impact and can be due to tissue resistance (Smit & Dubery, 1997), increase in phytoalexin synthesis (Mace *et al*., 1976), increase in enzymes and protein contents (Zhou, 2006) or incorporation of antimicrobial genes in the host plant (Rajasekaran *et al*., 2005). Up till now only one resistant locus named *Ve* locus has been identified from tomato (Fradin *et al*., 2009) against *V. dahliae*. However, resistance in *G. hirsutum*, the main cultivated cotton in the world is highly limited. Breeding efforts have led to the development of tolerant cotton cultivars by incorporating genes from resistant germplasm but most of these have shown resistance to non-defoliating (ND) isolates and suffered severe damage from the defoliating (DF) isolates (Bell, 1994). Therfore, identification of highly resistant resources is necessary to manage *Verticillium* wilt at commercial level. In this scenerio, there should be some swift, cost effective and less time consuming approaches for the evaluation of disease resistance which is laborious and time-consuming in the field (Jansky & Rouse, 2000). In one of the previous study (Bolek *et al*., 2005), injecting spore suspension into stem of healthy seedling produced wilt symptoms after two to three weeks of inoculation but results were not consistent, varied with environmental fluctuations and may result in the spread of pathogen. During the process of systemic infection, fungus colonizes the xylem and spread conidia in the above ground parts with water stream. This will result in easily scoreable symptoms of chlorosis and necrosis which may lead to plant wilting and finally plant death (Perry & Evert, 1983) depending on the virulence of the fungus strain used. Several plant pathogens such as the genera *Aspergillus*, *Penicillium*,

Fusarium, *Claviceps* and *Alternaria* produce phytotoxins in liquid culture (Svabova & Lebeda, 2005); which have been successfully applied to accelerate the disease resistance evaluation in several crops such as banana, carnation, grapevine, strawberry and wheat (Svabova & Lebeda, 2005). Similarly, use of fungus culture filtrates (CFs) of *V. dahliae* can produce plant wilting and desication in relatively short period of time (Bae *et al*., 2011).

The present study is planned to develop efficient, reliable and robust methods for the screening of cotton germplasms against *Verticillium* wilt by comparing the pathogen and pathogen free approaches for inducing disease symtoms.

Material and Methods

Plant material and fungal strains: Seeds of *Gossypium hirsutum* Cv Zheda B, were surface sterilized (Wang *et al.*, 2011) and sown in water saturated vermiculite in germination boxes (28 cm x 14 cm x 14 cm). Three different cotton isolates of *V. dahliae* like V08sn-1 (isolated from Jiangsu province of China), Anyang (ACCC no. 36207, a popular *V. dahliae* strain from Anyang City, Henan Province, China) and V07df2 (ACCC no. 38084, isolated from Dafeng City, Jiangsu Province, China) were used in this study. Fungus from stocked cultures was activated on potato dextrose agar (PDA) plates containing ampicillin (50 mg/L).

Soil inoculation with different isolates of *V. dahliae*: To multiply *V. dahliae* for soil inoculation, barley seeds were boiled and autoclaved in 500 mL conical flasks at 121^{o}C for 30 min. After cooling overnight at room temperature, bottles were inoculated with 2-3 discs of PDA media carrying fungus mycelium and were kept at 28^{o}C for 7-10 days. Earthen pots (30 cm diameter x 40 cm high with a drainage hole at bottom) were filled with soil (clay-loam soil, organic matter content 25.4 g/kg) already mixed with *V. dahliae* (1 g fungus mycelium on barley seeds / kg soil). For control treatment soil was not mixed with fungus. Pots were well watered to create humidity to spread fungus in the soil. Three weeks old seedlings were transplanted in these pots and plants were grown in net house during cotton growing season of Hangzhou, China. Experiment was arranged in a randomized completely block design and contained five replicates against each isolate of *V. dahliae*, ten plants per replicate. Plants were watered after interval of every three days and disease indexes were recorded after 40 days of inoculation. Degree of infection caused by *V. dahliae* was determined by scoring leaf necrosis and vascular browning and their average was presented as disease index. The following standard formula was followed to calculate disease index (Tian *et al*., 2010).

$$\text{Disease index} = \frac{(\Sigma \text{ disease score} \times \text{number of infected plants})}{\text{Total checked plants} \times \text{highest grade disease (4)}} \times 100$$

Effect of Vd toxin on radical growth and detached leaves of cotton: In a parallel experiment conducted in laboratory, above mentioned isolates of *V. dahliae* were again sub cultured on freshly prepared (PDA) plates supplemented with ampicilin (50 mg/L) at 28^{o}C in dark for 5 days. Then 2-3 circular disks (1 cm^2 each) of PDA carrying fungus mycelium were inoculated into 500 mL Czapek media (3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 1.0 g $MgSO_4$. $7H_2O$, 1.0 g KCl, and 0.01 g $FeSO_4$. $7H_2O$ in 1 L of distilled water) containing 3% sucrose and kept for continuous shaking at 170 rpm and 28^{o}C for 21 days. The Vd toxin from liquid cultures was isolated according to previously described method (Zhen & Li, 2004). Briefly, fungus cultures were filtered through four layers of muslin cloth and filtrate was centrifuged at 10, 000 *g* for 20 min. The supernatant was collected into another bottle and stored as crude Vd toxin at 4^{o}C for future use. Total protein contents of toxin solution were determined according to (Bradford, 1976) while total soluble sugars were measured as described by (Yemm & Willis, 1954) using bovine serum albumin and glucose as standards, respectively.

To monitor the bioactivity of the toxin, cotton seeds were germinated on water moistened filter paper in germination boxes. After 48 h, germinated seeds with emerged radicals were treated with 10 µg/mL of toxin and the data were recorded after 72 h. There were five replicates and 30 seeds per replicate arranged in completely randomized design. Data represent the mean values of radical from 150 seeds. Similarly, leaves (third uppermost) from 45 days old plants were detached from the main plant and kept on two layers of water moistened filter paper in petri plates. About 1 mL of toxin solution was added on different spots of the dorsal surface of the leaf. For control treatment double distilled water (DDW) was used instead of Vd toxin. Plates were sealed with parafilm, kept at 25-30 ^{o}C temperature and 16 h light/8 h dark period and symptoms were recorded after 72 h.

Confirmation of the elicitor component of the VD toxin: To confirm the elicitor component of crude Vd toxin, leaves were detached from the main plant and dipped via their petioles in 2 mL centrifuge tubes containing original toxin solution from Anyang isolate and an equivalent quantity of glucose (55 mg/L) in another set of tubes. The solution on the basis of need was renewed and data were recorded after 72 h.

Statistical analysis

The analysis of variance was conducted between different isolates. The significant differences between control and different isolates of *V. dahliae* were worked out by LSD multiple range tests ($p<0.05$) using the SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Origin Pro 8.0 version (Origin lab corporation, Wellesley Hills, Wellesley, MA, USA) was used to prepare graphs.

Results and Discussion

Generally speaking, *V. dahliae* isolates infecting cotton can be classified into defoliating type (D) and non defoliating (ND) pathotypes (Bejarano-Alca´zar *et al*., 1995, 1996). Chlorosis is the most common symptom observed after inoculation of the plant with D or ND type of *Verticillium dahliae* and results in stunted growth of

the plant. However, in case of ND type leaves became necrotic but remained attached to the stem while D type results in defoliation (Göre *et al*., 2009). Thus ND pathotype is moderately severe in contrast to highly virulent D type in cotton (Schnathorst & Mathre, 1966; Schna-thorst *et al*., 1975).

In present study, plants growing in non inoculated soil were healthy with fully expanded green leaves. But, inoculation of soil with different isolates of *V. dahliae* resulted in stunted growth of the plant, chlorosis and necrosis of leaf, which are the peculiar symptoms of *Verticillium* wilt. Chlorotic symptoms begin to appear from leaf margins and progress to the centre of the leaf. At later stages, this chlorosis turned into necrosis and resulted in curling of the leaf (Fig. 1). Moreover flowering was also delayed in infected plants as compared to their non infected control. Similar symptoms were observed by Bolek *et al*., (2005) upon injection of the cotton stem with conidial suspension of *V. dahliae*.

Observation of the symptoms is the most commonly used method to evaluate VW resistance in large breeding populations (Bae *et al*., 2010) and severity of attacks by *V. dahliae* depends upon its virulence (i.e., the amount of disease caused in a host genotype) of the pathogen isolates (Bell, 1994). Present study also revealed significant differences for the degree of virulence among different isolates of *V. dahliae* (Fig. 2). Highest disease index was 65 and scored for V08sn-1 (highly virulent) followed by 40 for Anyang isolate (moderate virulent) and 27 for V07df2 (less virulent). The results can be also visualized from the leaf necrosis and vascular browning which is in order of V08sn-1 > Anyang > V07df2 (Fig. 2). In current study, significant differences were observed for protein contents of these isolates (Fig. 3). Highest contents of soluble proteins were recorded for V08sn (78 mg/L) followed by Anyang (55 mg/L) and V07df2 (43 mg/L).

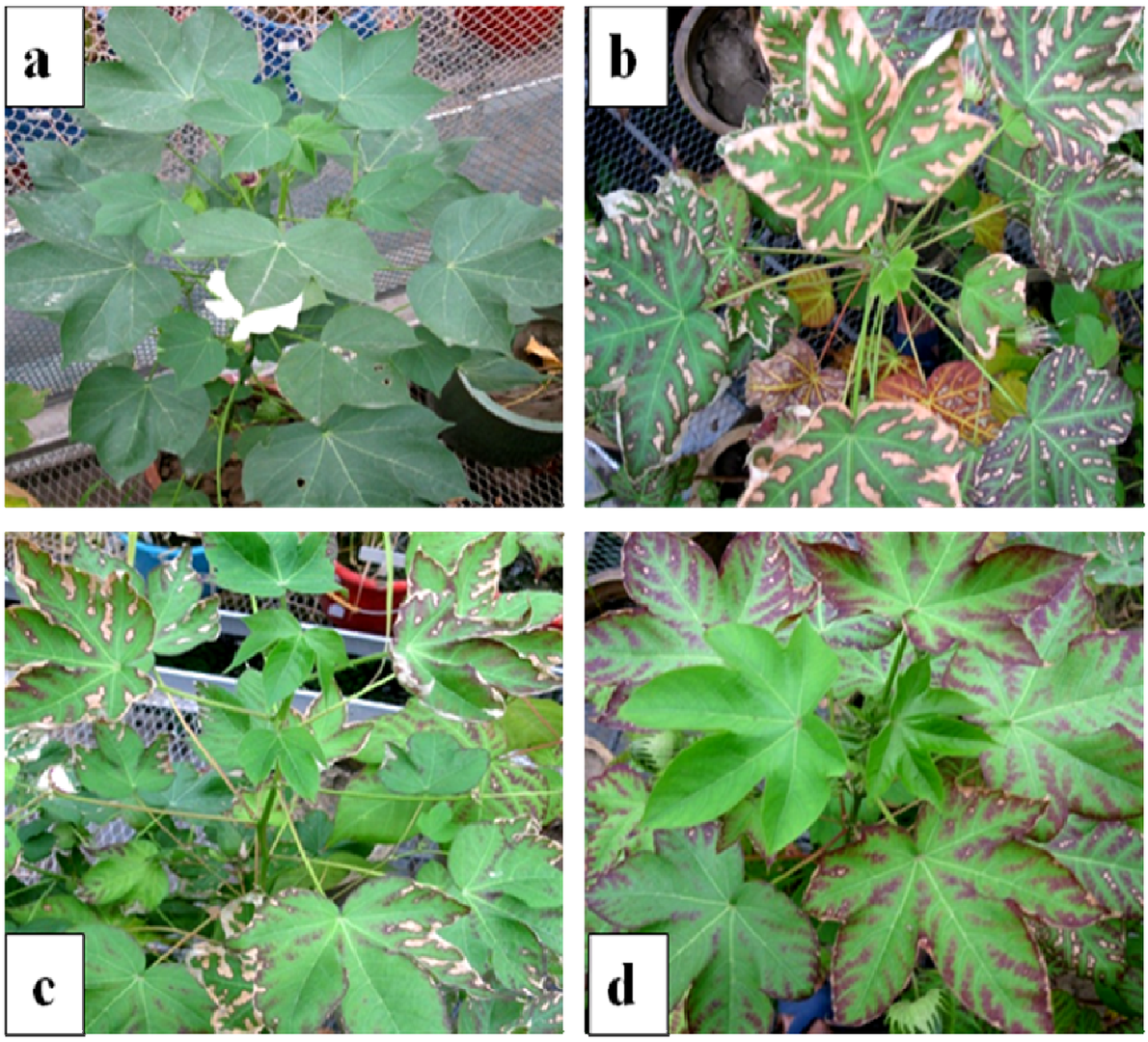

Fig. 1. Chlorosis and necrosis in leaves of upland cotton induced by different isolates of *V. dahliae* in artificially inoculated pots three weeks after inoculation. a; Control (non inoculated soil), b; soil inoculated with V08sn-1 showing high degree of virulence by developing high level of necrosis and leaf curling, c; Anyang isolate showing moderate while, d; V07df2 less virulent with low level of necrosis.

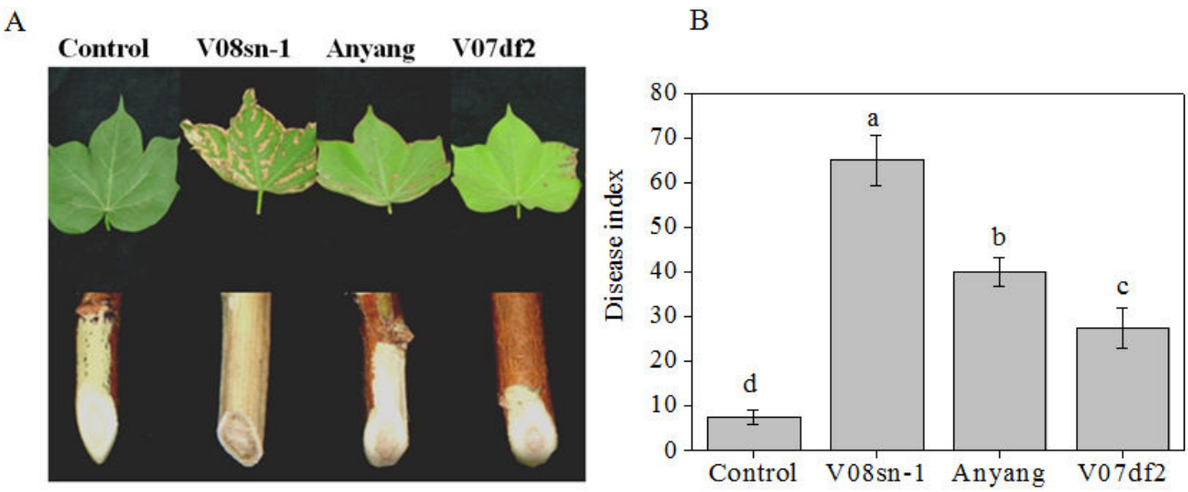

Fig. 2. Effect of different strains of *V. dahliae* on leaf necrosis and vascular browning (A); Disease index calculated as an average of leaf necrosis and vascular browning (B). Note: There were five replicates for each fungus isolate and ten plants per replicate. So data overall present mean data of 50 plant against each strain.

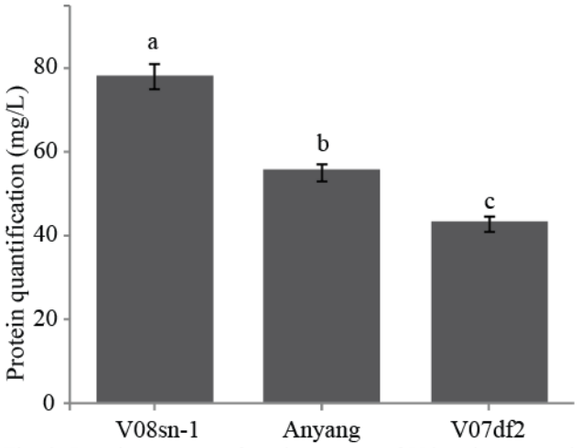

Fig. 3. Protein contents of crude extracts of Vd toxin prepared from various strains of *V. dahliae.* Note: Data presents mean value from five replicates against each isolate of *V. dahliae*.

Fungal culture fitrates (CFs) are presumed to contain phytotoxins distinctive to pathogen and have been used previously to study the early defense mechanisms in plant pathogen interactions (Davis *et al.,* 1998). This kind of pathogen free model system was also employed previously (Zhaoqing *et al.*, 1999) who reported that glycoproteins of CFs of *V. dahliae* (Vd-toxin) can induce cotton wilting syndrome.

To find a relationship for disease severity between fungus pathogen (*V. dahliae*) in soil inoculation experiment and the liquid culture (Vd toxin) of above mentioned three isolates, a parallel experiment was conducted using Vd toxin solution on germinated seeds and detached cotton leaves. Vd toxin treatment decreased the radical growth as compared to the non treated healthy control and the magnitude of this decrease was significantly different among different isolates with higher inhibitory effect seen for V08sn (62%) followed by Anyang (33%) and 17% in V07df2 (Fig. 4). Similarly, treatment of cotton leaf with Vd toxin produced marked symptoms of chlorosis as compared to control leaf. In addition, V08sn (highly virulent) and Anyang (moderate virulent) also produced necrotic patches in addition to chlorosis while no necrosis was observed when leaf was treated with toxin isolated from less virulent strain V07df2. This necrosis can be due to *V. dahliae* necrosis- and ethylene-inducing protein (VdNEP) component of Vd toxin (Wang *et al*., 2004) which acted as a defense elicitor rather than a pathogenicity factor and leads to the programmed cell death. The results of toxin bioactivity were consistent with pot inoculation experiment and a necrotic patch was larger in V08sn-1 treatment than Anyang isolate (Fig. 5). Bae *et al.,* (2010) also inoculated the potato roots with spore suspension of *V. dahliae* and Vd toxin. Their finding were similar to our study that Vd toxin caused earlier onset of symtoms as compared to the spore suspension.

V. dahliae when cultured in Czapek media produces carbohydrates in addition to protein so to rule out the contaminating effects of carbohydrates with proteins in the crude preparation of Vd toxin and to confirm the elicitor component of toxin solution causing leaf wilting, leaf petioles were either dipped in original toxin solution of Anyang isolate or an equivalent quantity of glucose (55 mg/L). It is reported (Meyer *et al.,* 1994) that Vd toxin contain 15.7% protein, 13.0% lipid, 0.4% phosphate and 70% carbohydrate. Later, this elicitor was purified by Davis *et al.*, (1998) and found to be a 65-kDa glycoprotein. Later on only the protein component (53-kDa deglycosylated protein was found to be responsible for the elicitor activity. Results showed that leaves dipped in glucose solution were highly turgid and fully green while leaves dipped in toxin solution initially resulted in leaf wilting, and finally leaf desiccation (Fig. 6). Similar wilting and leaf desiccation was observed (Wang *et al.*, 2004a) upon dipping leaf petiole in crude extract of Vd toxin and purified (VdNEP). Based on this result, toxin for future studies will be diluted on the basis of protein content. Thus utilization of Vd toxin can be an alternate of directly using fungus pathogen *V. dahliae* for the screening of the cotton germplasms against Verticillium wilt. This will not only accelerate the process of screening but will also offer an environmental friendly approach preventing the spread of the pathogen.

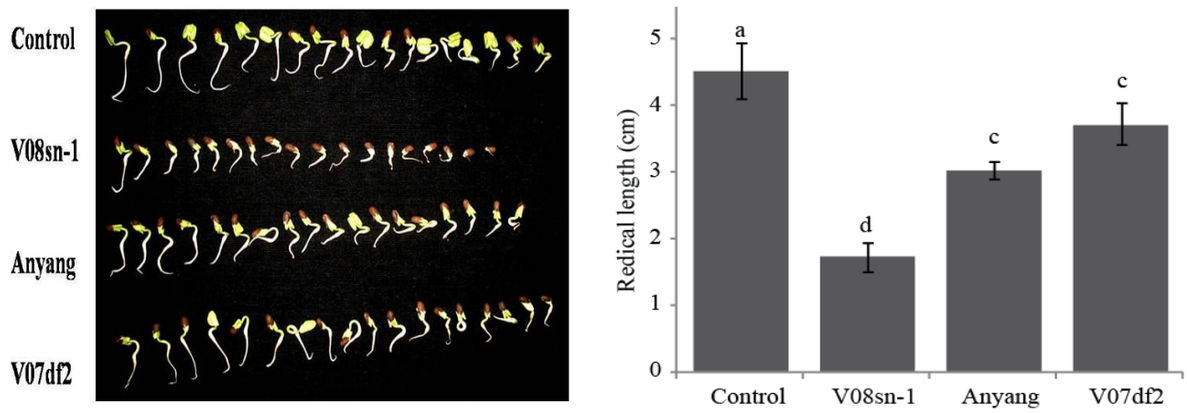

Fig. 4 The effect of three different *V. dahliae* toxins on radical length from germinating cotton.

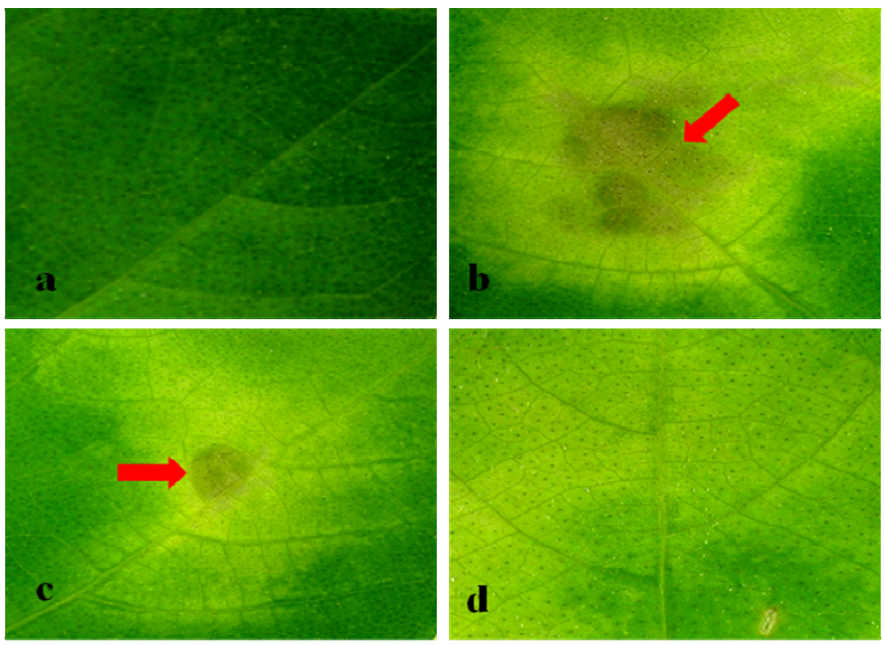

Fig. 5. Hypersensitive response in detached cotton leaves induced by Vd toxin from different strains of *V. dahliae* 72 hrs after infiltration a; is negative control treated with double distilled water (DDW), b; highly virulent V08sn-1, c; moderate virulent Anyang isolate and d; less virulent V07df2. Leaves were detached and placed on water moistened filter paper in petri plate. After that 1 mL of respective toxin solution or DDW was added on abaxial surface of leaf, plates were closed using parafilm and data were recorded after 72 hrs.

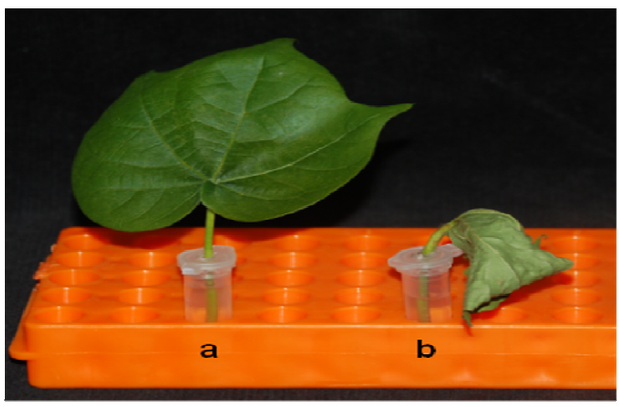

Fig. 6. Effect of filtered culture of Anyang isolate of *V. dahliae* on leaf wilting by dipping leaf petiole in 55 mg/L of glucose solution (a) and original toxin solution (b). Leaf dipped in glucose solution remained alive while leaf treated with toxin solution inititally wilted and then show desiccation after 72 hrs.

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