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IMPACT OF *VERTICILLIUM DAHLIAE* TOXIN ON MORPHOGENETIC, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF UPLAND COTTON

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Abstract

To obtain insights into the mechanism of *Verticillium* wilt, we elucidated the effects of *Verticillium dahliae* toxin (Vd toxin) on physiological and biochemical responses of the upland cotton (*Gossypium hirsutum* L.). Our results demonstrated that Vd toxin significantly ($P<0.05$) decreased the nutrition uptake up to 75% in NIAB-846, 70% in Zheda B, 63% in Zheda R, 56% in NIAB-111 and 34% in NIAB-999 than non-treated control. Significant reduction was observed in root growth parameters like root surface area, root length, root diameter and root volume. Further, Vd toxin treatment triggered the levels of superoxide dismutase (SOD), while reduced the contents of ascorbate (ASA) and reduced glutathione (GSH). However, the magnitudes of decrease in antioxidants values were lower in NIAB-999 resulting relatively low accumulation of malondialdehyde (MDA) as compared with other genotypes. In addition, the toxic effects of 24 h Vd toxin treatment were persistent even after two weeks of its removal and resulted in suppressed plant growth. According to integrated score, cotton genotypes were categorized for relative tolerance against *Verticillium* wilt in an order of NIAB-999 > NIAB-111 > Zheda R > Zheda B > NIAB-846. Our findings indicated that utilization of Vd toxin is environmentally friendly approach and nutrition uptake by plant might be a valuable tool for the screening of cotton genotypes against *Verticillium* wilt.

Key words: Biotic stress, nutrition uptake, root architecture, toxin, upland cotton**,** *Verticillium dahlia.*

Abbreviations: ASA; reduced ascorbate, GSH; reduced glutathione, MDA; malondialdehyde, PDA; potato dextrose agar, SOD; super oxide dismutase, Vd; *Verticillium dahliae*.

Introduction

Plant pathogens usually use more than a few mode of actions to overcome the mechanical, chemical or physiological barriers to penetrate into the host tissues. *Verticillium dahliae* is a soil born fungus which causes enormous yield losses in economically important crop plants including cotton. The fungus colonizes the xylem tissue by massive growth of its mycelium and form tyloses due to degradation of the plant cell wall components through secreting cell wall degrading enzymes (VanderMolen *et al*., 1983). This result in decreased hydraulic conductance to the above ground plant parts and a condition of water deficit is created which further leads to plant wilting, a symptom associated with the *Verticillium* wilt.

Gossypium hirsutum L., the main cultivated cotton around the world, is highly sensitive to the *V. dahliae* infestation. Though, the variation in relative tolerance to *Verticillium* wilt have been observed among different cultivars but resistance in upland cotton is highly limited (Zhang *et al*., 2012). Besides, commonly used cultural practices are futile to control the disease due to the absence of host specificity and extreme variability of pathogenicity. Similarly, most of the currently available fungicides are unproductive and their continuous use will not only result in the resistant pathotypes but also escalate the environmental pollution (Fradin & Thomma, 2006). Furthermore, a great number of other strategies *viz.* seeds treatments with known strains of *Pseudomonas* spp. and *Serratia plymuthicaca* (Erdogan & Benlioglu, 2010), *Trichoderma virens* (Hanson, 2000) and the use of organic amendments in the field (Huang *et al.,* 2006) though reduced the yield losses but none of these approaches could recover the desired targets of crop yield.

In this scenario, breeding of resistant cultivars would be the solitary approach to manage the disease. Unfortunately, up till now only one resistance locus *Ve* has been cloned from tomato (Kawchuk *et al*., 2001) and most of the tolerant varieties have shown resistance to non-defoliating isolates and suffer inexorable damage from the defoliating isolates of *V. dahliae*. Therefore, conventional breeding for improvement of cotton resistance by incorporating genes from resistant germplasm has not been successful and identification of new resistant lines or development of genetically engineered cotton is imperative to achieve enhanced resistance against known pathotypes of *V. dahliae.* Indeed, a great research has been carried out to evaluate the relative tolerance of various genotypes using different approaches, for instance; stem injection (Göre *et al*., 2009), root dip inoculation, leaf pricking and leaf petiole dipping (Wang *et al*., 2004; Bibi *et al.,* 2013a) using spore suspension and fungal toxins. It is speculated that pathogen produce toxins to cause an extreme level of disease, therefore screening of the crop plants against pathogen derived toxins will offer identification of highly resistant or tolerant cultivars than pathogen itself. Moreover, toxins have the ability to develop easily monitored symptoms of chlorosis, necrosis, and growth inhibition on healthy plants in relatively less time duration. A great number of previous studies utilized crude *V. dahliae* toxin (Vd toxin) and successfully achieved the characteristic symptoms of *V. dahliae* infection (Jiang *et al*., 2005, Wang *et al*., 2004, Zhen & Li, 2004, Bibi *et al.,* 2013a, Li *et al.,* 2016).

Despite recent advances in elucidation of *Verticillium* wilt, there are still significant gaps in our understanding the effects of Vd toxin on plant biochemical responses and their relation with the plant wilting syndrome in cultivated cotton. Besides, the lingering effects of Vd toxin on plant growth and host defense response is still unclear. Therefore, further studies are necessary to provide a detailed description of the extent and mechanism of Vd toxin induced stress. The present study is aimed to understand the morphogenetic, physiological, and biochemical aspects of cultivated cotton in response to Vd toxin treatment; and is critical to provide a basis for developing strategies to maintain a sustainable cotton production.

Material and Methods

Fungus growth and toxin preparation: Anyang isolate of *V. dahliae* (complete reference cited by Ni *et al*., 2013) was sub-cultured on Potato Dextrose Agar (PDA) and then 2-3 circular disks (1 cm^2 each) of PDA carrying fungus mycelium were inoculated into 0.5 L 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°C for 21 days. Toxin from liquid culture was isolated as described previously (Zhen & Li, 2004) and its total protein contents were determined using bovine serum albumin as standard (Bradford, 1976).

Plant growth conditions, treatment method and nutrition uptake: Five cotton genotypes from two different origins were used to observe genetic variations for their relative tolerance against *Verticillium* wilt. Seeds of Zheda B (maintainer) and Zheda R (restorer) were collected from College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China, while NIAB-111, NIAB-846 and NIAB-999 were native to Pakistan and collected from the Nuclear Institute of Agriculture and Biology (NAIB), Faisalabad, Pakistan. Hydroponic culture was used for the growth of plants in the growth room with condition set at temperature of 28±2 °C and 16 h light/8 h dark period. Fifteen days old seedling of the above mentioned genotypes were transferred to plastic pots (one plant per pot) filled with basic nutrient solution (BNS) (Ahmed *et al*., 2013). The experiment was arranged in a completely randomized design with five replicates (one plants per replicate). After forty five days of growing, BNS solution in pots was replaced with BNS + Vd toxin (100 mg L^{-1}) and the total weight of the pot along with plant was measured. Following 24 h of Vd toxin stress treatment, that induced stress at cellular level in our previous study (unpublished work), pots were weighed again and the difference between two values was taken as nutrition uptake. The BNS + Vd toxin solution was removed, roots were thoroughly washed with sterilized water and plants were grown in fresh pots containing only BNS solution and kept for two weeks of recovery period. Control and treatment solutions were changed after every two days to avoid the metabolic effects of plant root exudates. After two weeks, nutrition uptake was again measured and plants were harvested to observe the effects of Vd toxin treatment on plant growth parameters and root architecture. For various biochemical and physiological analysis, leaf samples were collected at two different timings i.e. after 24 h of Vd toxin treatment (stress) and two weeks of recovery period (recovery).

Measurement of chlorophyll fluorescence: Chlorophyll fluorescence was measured with a PAM-2100 pulse modulated fluorometer (Walz, Effeltrich, Germany). Leaves were kept in dark for 20 min and then were illuminated under a high saturating light pulse with frequency of 0.05 Hz for 260 s. The initial fluorescence (F_o) was determined using a measuring beam, the maximal fluorescence (F_m) was determined using saturating pulse (2500 µmol m^{-2} s^{-1} PAR). Variable florescence (F_v) was calculated from the formula: $F_v = F_m - F_o$. Maximal photochemical efficiency of PS II (F_v/F_m) was calculated as described previously (Ahmed *et al*., 2013).

Determination of lipid peroxidation, SOD activity and analysis of ASA, GSH contents: About 0.5 g of leaf material was ground to fine slurry in 5 mL of 50 mM phosphate buffer (PBS, pH 7.8) using a pre-chilled mortar and pestle. Homogenates were then transferred into 10 mL tubes and centrifuged at 10,000 *g* for 15 min. Supernatants were collected into fresh tubes and stored at -70°C for analysis of biochemical parameters.

To measure lipid peroxidation product malondialdehyde (MDA), 1.5 mL sample was taken in 10 mL tube and heated along with 2.5 mL of reaction solution (0.5% TBA dissolved in 5% TCA solution) at 95°C for 30 min. The reaction was stopped by taking out the tubes from water bath and immediately keeping on ice. Tubes were centrifuged at 8000 *g* for 10 min and supernatant was used to measure absorbance at 532 and 600 nm. MDA content was calculated as nmol/g FW.

For analyzing superoxide dismutase (SOD, EC 1.15.1.1) activity, 2.725 mL of reaction solution [75 µM/L NBT, 20 µM/L riboflavin, 100 µM/L EDTA-Na_2, 25 µL of enzyme, 130 mM/L methionine] and 0.25 mL ddH_2O were taken in 25mL glass beaker. Reaction was started with addition of 25 µL of enzyme and exposing beakers to high intensity light of about 4000 lux for 20 min. Control were also treated in a similar way except addition of enzyme. SOD activity was calculated by measuring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) as described previously (Bibi *et al.,* 2013b) and one unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT as monitored at 560 nm.

Contents of reduced ascorbate (ASA) were determined according to previously described method (Ahmed *et al*., 2013) while reduced glutathione (GSH) was determined using a GSH colorimetric activity assay kit (Jiancheng Bio Co., Nanjing, China).

Determining the plant growth parameters and root architecture: After two weeks of recovery period, data for plants growth parameters like plant height (cm), fresh weight of leaves, stem and root were recorded. For estimation of dry weight, separated plant parts were kept at 80°C for 10 h until they attain constant mass. Whereas,

roots of control and toxin treated plants were used to monitor root architecture. For this purpose, roots were arranged in a 20 cm wide and 30 cm long acrylic container, containing approximately 1 cm of water and root automatism scan apparatus (MIN-MAC, STD1600+), equipped with Win RHIZO software offered by Regent Instruments Company. Data for average root length (cm), root surface area (cm^2), root diameter (mm) and root volume (cm^3) per plant were recoded. The analyzed according to previous researchers (Daud *et al*., 2009).

Statistical analysis

The analysis of variance was conducted between different treatments. The significant differences of five cotton genotypes between control and treatments 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

Effect of Vd toxin on root architecture and plant growth parameters: Growing conditions other than control significantly ($P<0.05$) decreased plant height, seedling fresh weight and individual leaf, shoot and root fresh/dry weights under toxin treatment. Genotypes significantly differed individually and significant interaction between genotype and growing conditions (G×T) existed for all traits (Table 1). Among five tested genotypes; the highest plant height was recorded in Zheda B, while lowest in NIAB-111. NIAB-999 was the genotype with the highest biomass (fresh/dry weights) and followed by NIAB-111; on the other hand, NIAB-846 exhibited the lowest biomass across the stressful treatments. Exposure of plants to Vd toxin induced stress for 24 h affected the normal root growth development in all evaluated genotypes. Significant differences ($P<0.05$) were observed among genotypes as compared to their respective control. Root surface area, root length, root volume and root diameter were decreased in order of 83%, 74%, 78%, 81% in Zheda R >73%, 62%, 27%, 62% in NIB-846 >60%, 59%, 60%, 53% in Zheda B> 55%, 56%,26%, 42% in NIAB-999 > 40%, 48%, 17%, 48% in NIAB-111, respectively (Fig. 5). Moreover, these parameters in addition to plant biomass were found to be significantly correlated with nutrition uptake (Figs. 1, 2).

Effect of *Verticillium dahliae* toxin (Vd toxin) on nutrition uptake and chlorophyll florescence: Treatment of the cotton plants with Vd toxin induced plant wilting within 24 h and significantly reduced the uptake of nutrition by plant roots in all genotypes. However, the percent reduction was significantly different among the genotypes as compared to their respective controls. After 24 h of stress, maximum reduction of 75% in nutrition uptake was recorded for NIAB-846 and followed by 70% in Zheda B, 63% in Zheda R, 56% in NIAB-111 and 34% in NIAB-999 as compared with non-treated healthy control. The toxic effects of Vd toxin treatment were persistent even after two weeks of recovery period and nutrition uptake was still lower than corresponding control in all genotype (Fig. 3). In comparison to the control, the highest percent reduction was recorded in order of Zheda R >Zheda B, NIAB-846 >NIAB-999 >NIAB-111 with values of 48, 47, 47, 30 and 18%, respectively.

Leaf chlorophyll florescence was evaluated in terms of F_v/F_m ratio and depicted in the Fig. 3. Results revealed a significant decrease in F_v/F_m value due to Vd toxin treatment in all genotypes as compared to their controls. After 24 h of stress, maximum decrease in *Fv/Fm* value was observed in NIAB-846 i.e. 19% lower than its control and followed by 12% in Zheda R; 8% in Zheda B, 10% in NIAB-111 and 8% in NIAB-999. Following two weeks of recovery period, reduction in *Fv/Fm* value was slightly alleviated in all genotypes (except Zheda R) but the magnitudes were still lower than control. The F_v/F_m value was 14% for Zheda R, 10% for NIAB-111, 7% for Zheda B, 3% for NIAB-999 and 1.7% in NIAB-846 lower than its control.

Table 1. Effect of Vd toxin treatment (100 mg L^{-1}) on plant growth parameters of five different genotypes cotton belonging to *G. hirsutum*.

Treatment	Plant height	Leaf FW	Stem FW	Root FW	Leaf DW	Stem DW	Root DW
Control	24.87 a	10.34 a	5.54 a	5.15 a	1.70 a	1.09 a	0.25 a
Toxin	19.53 b	2.48 b	3.27 b	2.72 b	0.49 b	0.49 b	0.11 b
Genotypes							
Zheda B	25.33 a	4.90 b	4.21 b	3.18 b	0.76 d	0.80 b	0.10 d
Zheda R	23.00 b	5.98 b	4.53 b	2.98 b	0.97 c	0.84 b	0.18 b
NIAB-111	18.16 c	10.32 b	5.18 a	5.21 a	1.06 b	0.99 a	0.21 b
NIAB-846	22.00 b	5.13 b	3.36 c	2.91 b	0.89 c	0.42 c	0.15 c
NIAB-999	22.50 b	13.73 a	4.73 ab	5.36 a	1.78 a	0.90 b	0.26 a
Treatment	67.37 ***	257.47 ***	70.32 ***	164.52 ***	260.19 ***	190.57 ***	151.86 ***
Genotypes	12.72 ***	16.49 ***	5.05**	34.69 ***	22.36 ***	20.26 ***	25.65 ***
Treatment × Genotypes	4.18 **	12.42 ***	23.08***	4.50 **	13.50 ***	4.78 **	24.79 ***

For each genotype values shown are the means of five replicates. The means followed by different letters were significantly different ($P<0.05$) by LSD test. The associated sum of squares and probabilities (**$P<0.01$; ***$P<0.001$) are shown.

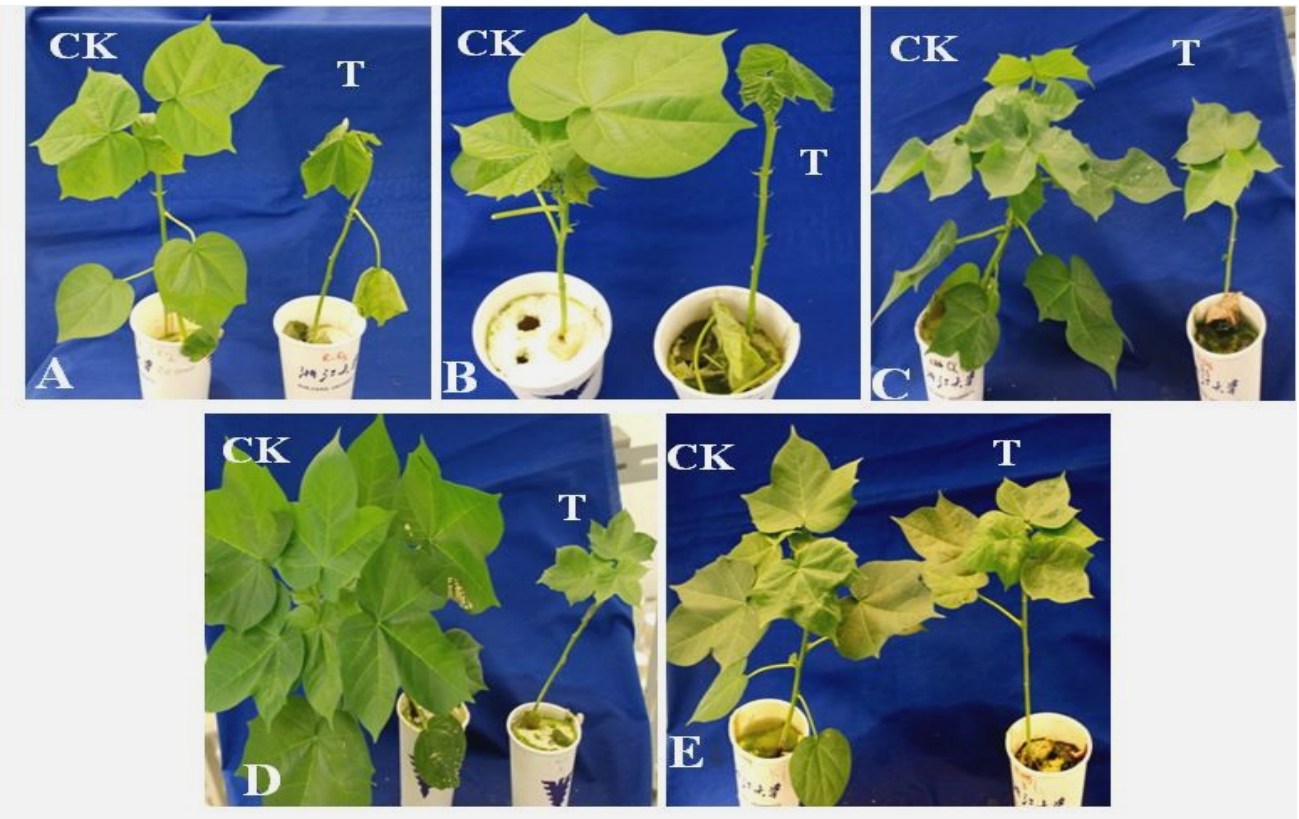

Fig.1. Effect of Vd toxin induced stress of *V. dahliae* on plant growth of different cotton genotypes.
Note: A; Zheda A, B; Zheda R, C; NIAB-111, D; NIAB-846, E; NIAB-999 while CK; Control plants, T; Vd toxin treated plants. Pictures were taken after two weeks of recovery period. Most of the leaves fell down from the plant as a result of 24 hrs of Vd toxin stress treatment, however, NIAB-999 still retained more leaves.

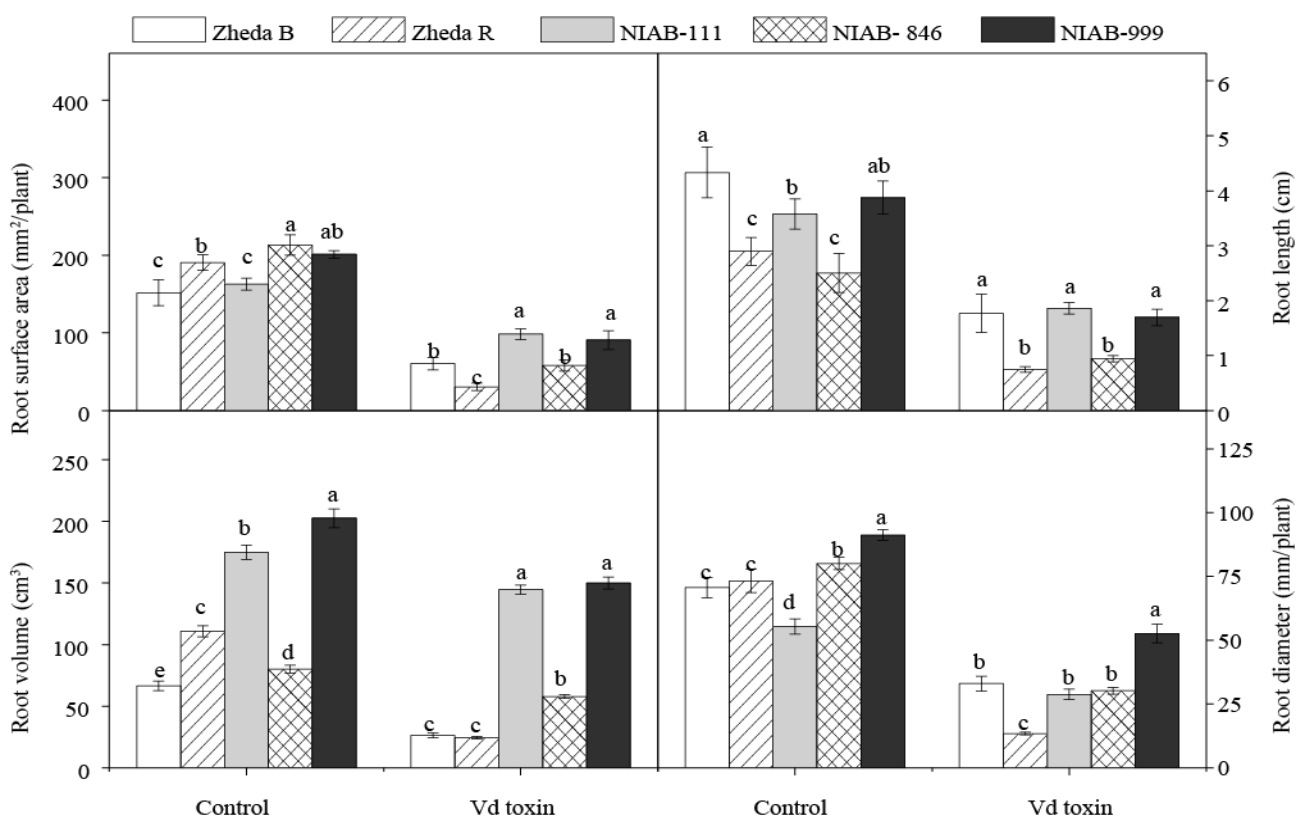

Fig. 2. Effect of Vd toxin (100 mg L^{-1}) induced stress of *V. dahliae* on plant root architecture of different cotton genotypes. Each value in the graph shows the mean with the standard deviation of five replicates. Different letters indicate significant differences ($P<0.05$) among the treatments.

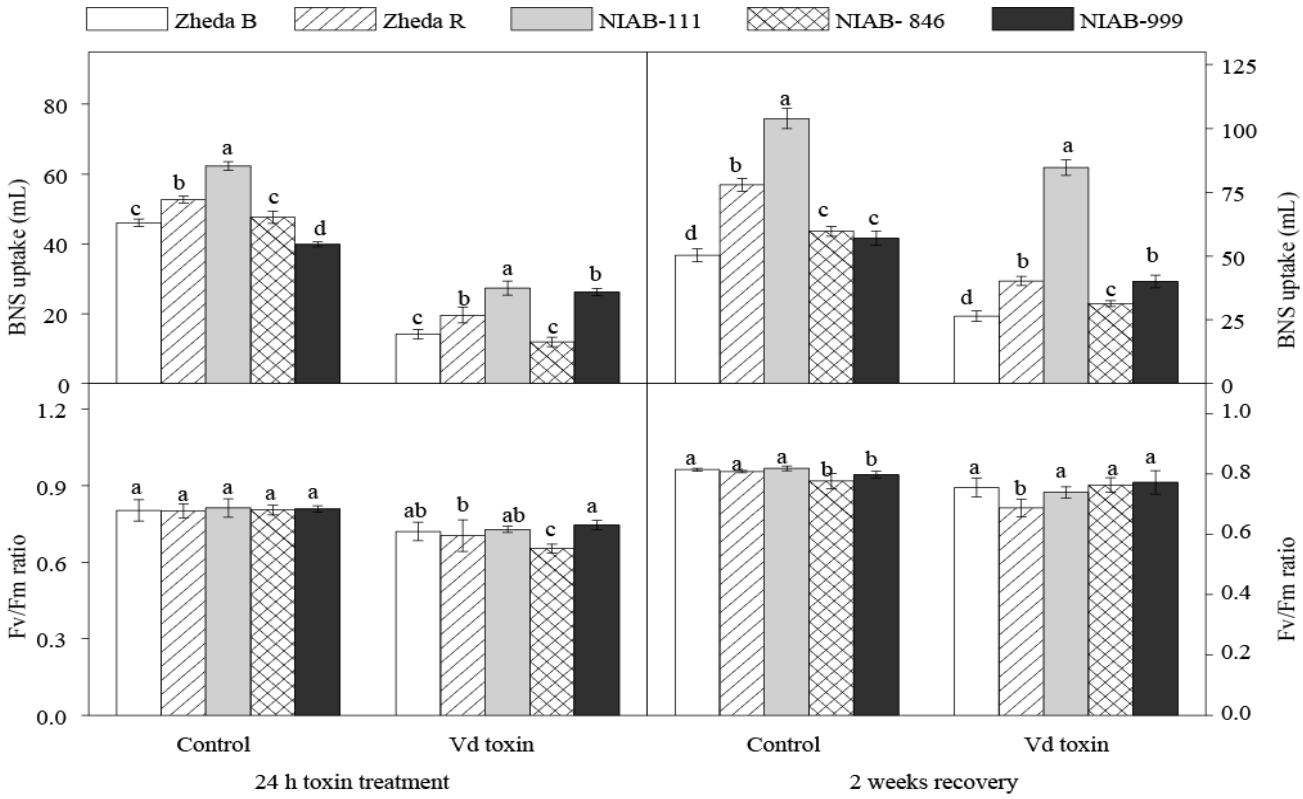

Fig. 3. Effect of Vd toxin (100 mg L^{-1}) induced stress of *V. dahliae* on plant nutrition uptake and chlorophyll florescence i. e. *Fv/Fm* ratio of different cotton genotypes after 24 hrs of stress application (left panel) and two weeks of recovery period (right panel). Each value in the graph shows the mean with the standard deviation of five replicates. Different letters indicate significant differences ($P<0.05$) among the treatments.

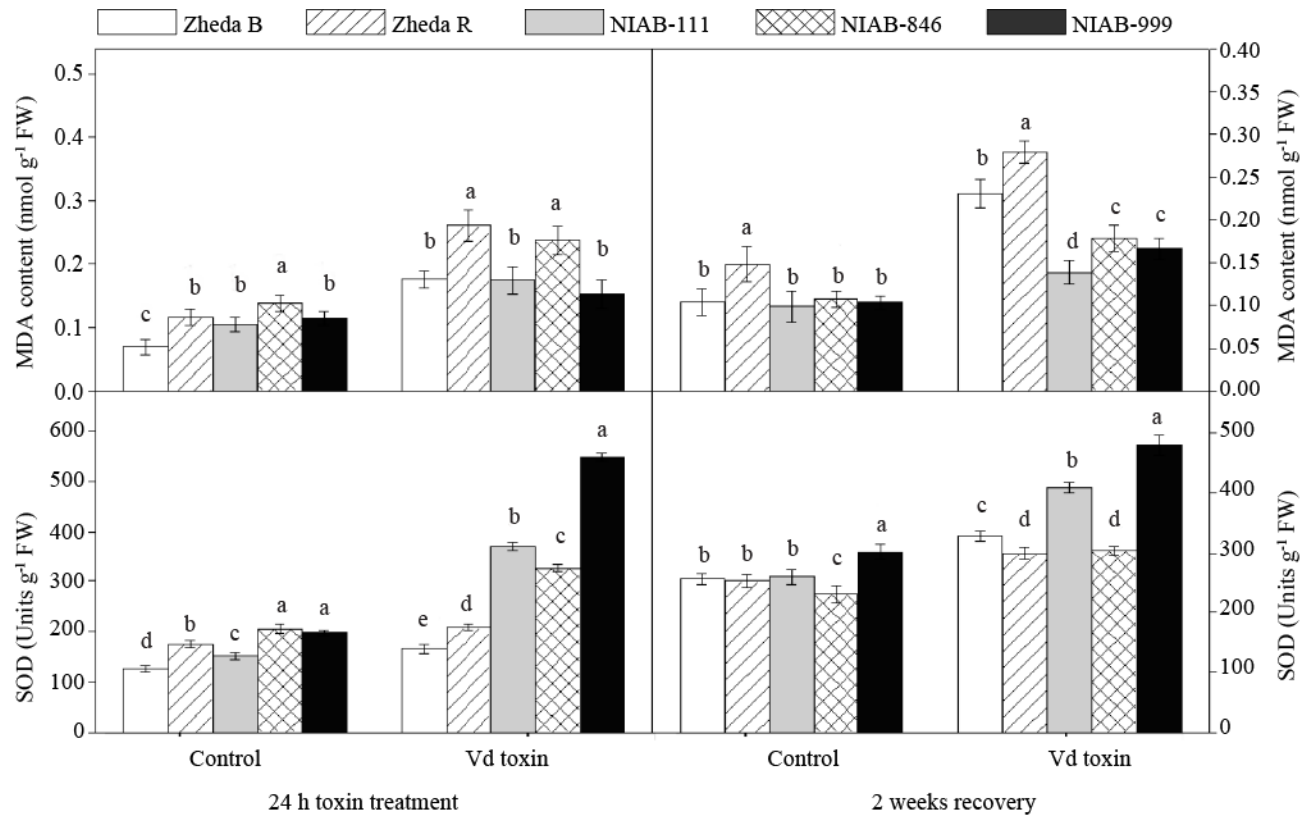

Fig. 4. Effect of Vd toxin (100 mg L-1) induced stress of *V. dahliae* on lipid per oxidation (MDA content) and superoxide dismutase activity (SOD) in leaves of different types of cotton genotypes after 24 hrs of stress application (left panel) and two weeks of recovery period (right panel). Each value in the graph shows the mean with the standard deviation of five replicates. Different letters indicate significant differences ($P<0.05$) among the treatments.

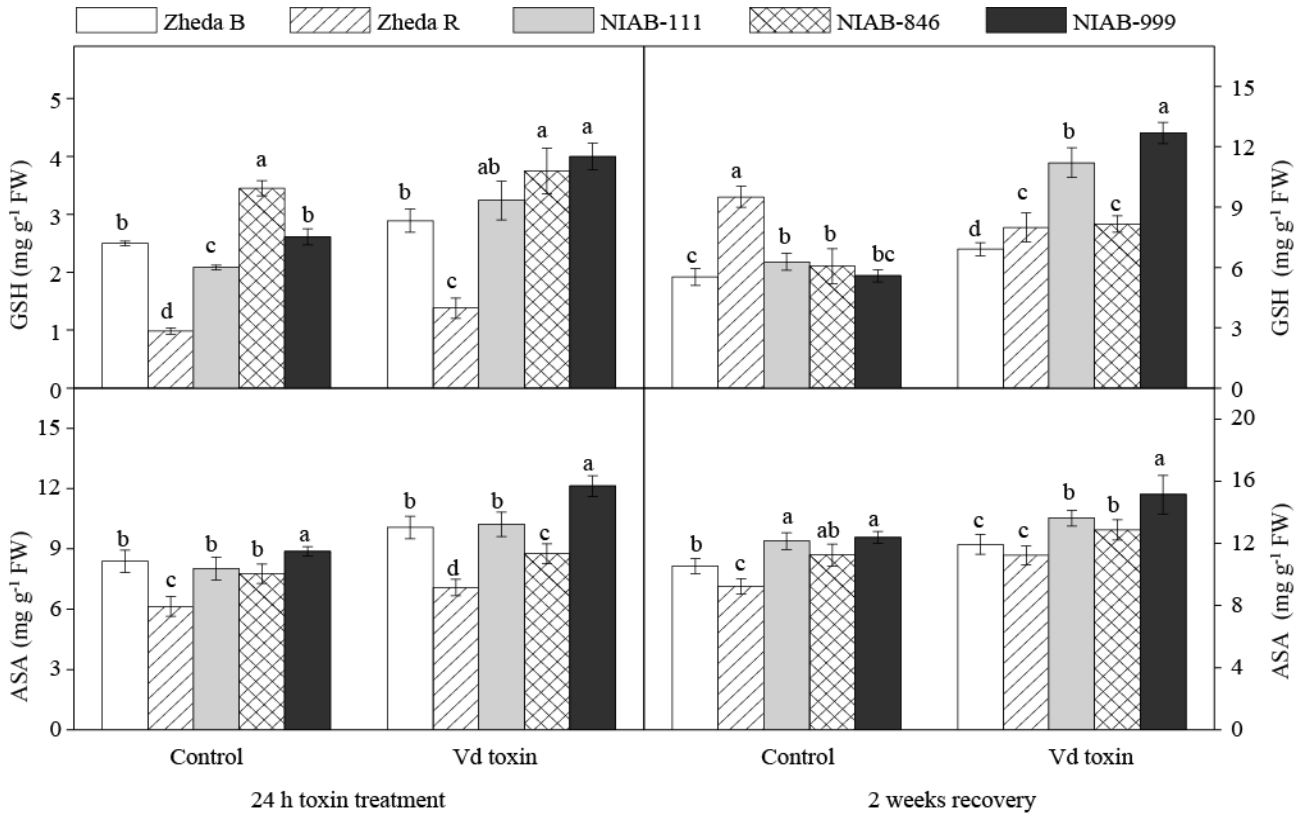

Fig. 5. Effect of Vd toxin (100 mg L-1) induced stress of *V. dahliae* on reduced glutathione (GSH) and reduced ascorbate (ASA) in leaves of different cotton genotypes after 24 hrs of stress application (left panel) and two weeks of recovery period (right panel). Each value in the graph shows the mean with the standard deviation of five replicates. Different letters indicate significant differences ($P<0.05$) among the treatments.

Changes in the contents of malondialdehyde (MDA) and activity of superoxide dismutase (SOD): The MDA contents were measured in cotton leaves as an indicator of oxidative stress. In our experiment, exposure of cotton plants to Vd toxin induced a significant increase in the quantity of MDA in all genotypes. After 24 h of stress application, the highest MDA contents of about 61% were observed in Zheda B, 55% in Zheda R, 41% in NIAB-846, 40% in NIAB-111, and 26% in NIAB-999. Furthermore, the MDA contents remained higher than control even after two weeks of recovery period which revealed that Vd toxin treatment have lingering effects. However, the magnitude of MDA increase was reduced in all genotypes except NIAB-999 as compared to MDA contents measured after 24 h. The accumulation of MDA contents after two weeks of recovery was 55%, 47%, 40%, 29% and 38% in Zheda B, Zheda R, NIAB-846, NIAB-999 and NIAB-111 higher than control, respectively (Fig. 4).

The activity of superoxide dismutase (SOD) changed significantly upon Vd toxin treatment. After 24 h stress, a dramatic increase in SOD activity was observed in NIAB-999 (63%) and NIAB-111 (59%) followed by 37% in NIAB-846, 23% in Zheda B and 16 % in Zheda R as compared to their control. However after two weeks of recovery period, the SOD activity was decreased but still higher than respective controls in all genotypes. The observed increment in SOD activity of NIAB-999, NIAB-111, NIAB-846, Zheda B, Zheda R was 37%, 37% 24% , 22% and 16% over their controls, respectively.

Changes in the contents of reduced glutathione (GSH) and reduced ascorbate (ASA): GSH contents were increased after 24 h of stress in all genotypes with maximum increase of 36% in NIAB-111 followed by 34% in NIAB 999; 29% in Zheda R; 14% in Zheda B; and 8% in NIAB-846. Compared with control, GSH contents remained significantly high even after two weeks of recovery period. The percent increase over control was in order of NIAB-999 >NIAB-111 >Zheda B >NIAB-846 >Zheda R with values of 56%, 44%, 41%, 26% and 13%, respectively. Contents of reduced ascorbate (ASA) were also influenced by Vd toxin induced stress. In general, ASA contents were increased after stress and stay high in all genotypes even after recovery as compared to their relative control. Highest increase of 27% over control was recorded in NIAB-999 followed by 22% in NIAB-111 while this increment was as low as 16% in Zheda B, 13% in Zheda R and 11% in NIAB-846, after 24 h of stress application. Furthermore, after recovery, ASA contents were high in all genotypes with greater effect in NIAB-999 (Fig. 5).

Discussion

Vessel occlusion is the primary cause of water stress in *Verticillium* wilt and might be either due to physical obstruction of the xylem tissue by pathogen itself or due to the host defense responses that are intended to prevent the spread of pathogen by vessel plugging. The utilization of Vd toxin as an elicitor of *Verticillium* wilt has been

successfully applied in cotton (Wang *et al*., 2004, Zhen & Li, 2004, Jia *et al*., 2007), tomato (Zhang *et al*., 2011), potato (Bae *et al*., 2011) and Arabidopsis (Jiang *et al*., 2005, Yuan *et al*., 2006) to accelerate the process of disease resistance evaluation. In previous studies, Vd toxin induced alteration of cytoskeletons and nucleoli in Arabidopsis suspension cells; production of nitric oxide in Arabidopsis leaves (Shi *et al*., 2012, Yuan *et al*., 2006), induced chlorosis and necrosis in potato plant (Bae *et al*., 2011) while stimulated cell wall lignification in cotton as a host defense response (Dubery & Smit, 1994). In our previous study (unpublished), we monitored the effects of Vd toxin at cellular and organ level of upland cotton (*G. hirsutum*) due to its known sensitivity to *V. dahliae*. Further, it was also logical to observe the residual effects of Vd toxin stress on morphogenetic, physiological and biochemical responses and to set a criterion for the evaluation of relative tolerance against *Verticillium* wilt in different cotton genotypes. This investigation not only broadened the understanding of the mechanism of Vd toxin induced stress but also provided a detailed knowledge about various strategies that could make cotton genotypes to overcome the prevailing stress.

Recognition of the host and to take control of its defense response is of prime importance for successful pathogen invasion. In contrast, cultivars resistant to *Verticillium* wilt often show decreases in the rate of the disease progress and the symptom severity with a lower percentage of foliar symptoms (Huang *et al*., 2006). This resistance can be due to physical barriers (Xu *et al*., 2011), increase in phytoalexin, enzymes and proteins synthesis (Mace, 1978) or incorporation of antimicrobial peptides in the host plant (Rajasekaran *et al*., 2005). Most of the vascular wilt pathogens reduced diameter of the conductive elements by their physical growth, producing metabolites or by inducing the formation of gummy substances and tyloses (Aguirreolea *et al*., 1995). Such vessel occlusion has been proposed as the primary cause of water stress in *Verticillium* wilted plants (Street & Cooper, 1984). In the present study, exposure of plants to Vd toxin resulted in a sharp and steep reduction of nutrition uptake after 24 h and this effect was persistent even after two weeks of recovery. Therefore, nutrition uptake by plants can be used as a direct measurement of plant wilting. After 24 h of Vd toxin treatment, the decrease in nutrition uptake was maximum in Zheda B (70%) while it was minimum in NIAB-999 (34%) than non-treated control. Even following two weeks of recovery, the nutrition uptake of toxin treated plants was lower than control and the magnitude of this decrease stands in an order of Zheda R > Zheda B, NIAB-846 >NIAB-999 >NIAB-111. The possible explanation of this reduction in nutrition uptake might be smashing of root tips in response to Vd toxin or closing of the stomata due to plant wilting which decreases the transpiration pull (Hampton *et al*., 1990). Besides, the chlorophyll florescence F_v/F_m was also significantly decreased in all genotypes due to Vd toxin treatment (Fig.3). However, the magnitude of the decrease in *Fv/Fm* value was not so high and might be due to the insensitivity of chloroplast to Vd toxin treatment (Zhen & Li, 2004). In addition, it has been also noted that ion channels of susceptible cotton cultivars are sensitive to crude extracts of *Verticillium* resulting in ion leakage so wilting symptoms might be due to disturbance in cell homeostasis caused by Vd toxin rather than because of vessel occlusion (Meyer *et al*., 1994). Therefore, it can be assumed that Vd toxin can suppress plant growth by interfering various metabolic processes, that may include inhibition of the proton pump, root growth, and damage to photosynthetic machinery.

Pytotoxins can act as suppressors of induced resistance and cause lipid peroxidation by generating reactive oxygen species (ROS). Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation and high MDA content always correlated with high level of oxidative stress. Once formed, this MDA can cause cellular damage by reacting with proteins, lipids and nucleic acids of cellular organelles. Maximum increase in MDA content was observed in Zheda B and can be associated with low activity of SOD, low contents of GSH and ASA. In contrary, relatively lowest levels of MDA in NIAB-999 might result from higher activities of antioxidants as compared to their control. Interestingly, after two weeks of recovery period the magnitude of MDA accumulation was decreased in all genotypes except NIAB-999 which exhibited relatively low and delayed effects of Vd toxin induced oxidative stress. Furthermore, MDA contents were higher than control even after two weeks of recovery period, hence demonstrate that plants are still facing oxidative stress due to residual effects of Vd toxin (Fig. 4).

The peculiar symptoms of *Verticillium* wilt includes leaf wilting, chlorosis, severe dwarfing, decrease in plant fresh and dry weight either due to the pathogen inoculation or Vd toxins treatment and were observed previously (Bae *et al*., 2011, Fradin & Thomma, 2006). In present study, Vd toxin treatment also suppressed the plant growth and reduced the plant height, fresh/dry weight of leaves, shoot and root in all genotypes with least reduction observed in NIAB-999 (Table 1). The decrease in fresh/dry weigh might be mainly due to leaf desiccation and leaf abscission as observed in our preliminary studies (Bibi *et al.,* 2013a) or due to decrease in photosynthesis as a result of stomatal closure in Vd toxin treated plants. Similarly, root surface area, root length, root volume and root diameter were also decreased in response to Vd toxin treatment with greater effect seen in Zheda R followed by NIAB-846, Zheda B, NIAB-999 and NIAB-111. Stress tolerance can be defined as enduring the effect of a pathogen infection while still producing a good crop yield which off course will be lower than healthy control and therefore tolerance can also be defined as partial resistance (Fradin & Thomma, 2006). To evaluate the relative tolerance of these genotypes against *Verticillium* wilt, we adopted the following formula-based integrated score = absolute values of [(plant height* × 0.2) + (plant fresh weight × 0.2) + (plant dry weight × 0.2) + (nutrition uptake × 0.2) + (*Fv/Fm* × 0.2) (*reduced (-) percentage in growth/physiological parameters relative to the controls as reported by (Ahmed *et al*., 2013). Thus, there is a negative correlation between Vd toxin stress tolerance of different cotton genotypes and the integrated score, i.e., the genotypes with the lowest and highest scores were considered as the tolerant and sensitive, respectively. According to the integrated scores, relative tolerance was in an order of NIAB-999 > NIAB-111> Zheda R> Zheda B > NIAB-846. (Fig. 1, Table 2).

In conclusion, comprehensive information has been provided on Vd toxin induced stress of *V. dahlia* and plant responses in upland cotton. It is proven that Vd toxin can be an appropriate and ecologically safe approach to monitor the plant pathogen interactions. Furthermore, it can inhibit the growth of the plant by affecting physiological and biochemical processes. Significant correlation among nutrition uptake and plant growth parameters proposed that nutrition uptake can be used as a best criterion for the screening of relative tolerance against *Verticillium* wilt. Similarly, increased levels of antioxidants can antagonize the residual effects of Vd toxin and favor the plant survival.

Table 2. Effect of Vd toxin induced stress of *V. dahliae* on plant height, plant fresh weight (g), plant dry eight (g), nutrition uptake and *Fv/Fm* of different cotton genotypes expressed as % decrease of control.

Genotypes	Plant height	Plant FW	Plant DW	Nutrition uptake	Fv/Fm	Integrtaed score
Zheda B	-3.54 ab	-12.57 bc	-11.8 b	-13.65 b	-1.95 bc	-43.53 b
Zheda R	-1.873 a	-13.75 bc	-14.09b	-16.27 c	-0.331 a	-46.33 b
NIAB-111	-2.144 a	-10.23ab	-13.79b	-15.65 c	-0.62 ab	-42.45 b
NIAB-846	-7.302 b	-15.84 c	-15.87 b	-8.054 a	-1.48 bc	-48.55 b
NIAB-999	-2.713 ab	-7.05 a	-3.09 a	-12.33 b	-2.98 c	-21.82 a
LSD.05 between genotypes	4.93	4.76	4.39	1.62	1.52	9.62

Different letters indicate significant differences (P<0.05) among 5 genotypes within each treatment. Integrated score=absolute values of [(plant height ×0.2) + (plant fresh weight × 0.2) + (plant dry weigh×0.2) + (nutrition uptake×0.2) + (*Fv/Fm* value ×0.2). (*decrease (-) percentage in growth/physiological parameters relative to controls)

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