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Stomata and root traits regulate drought adaptation in cotton plants

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Abstract

The fine regulation of root and stomatal architecture and physiological responses in crop plants towards drought stress is reported in the present study. The impact of water stress on relative water content (RWC), chlorophyll content, stomatal index and plant growth traits were evaluated in the two contrasting cotton varieties i.e., LRA-5166 (drought-tolerant) and NBRI-67 (drought-sensitive) during water stress. Results exhibited the significant variation in RWC, root and shoot length, root dry weight as well as an alteration in stomatal index between LRA-5166 and NBRI-67 subjected to water stress. Under water stress shoot dry weight, total dry weight and chlorophyll content in LRA-5166 were not found altered while it reduced in NBRI-67. Collectively, we demonstrated the two varieties of cotton behave differently to maintain biomass and withstand water stress. Moreover, we suggested that the physiological roles of increased root growth with reduced stomatal index distinctly maintain water homeostasis in LRA-5166 and NBRI-67 under water stress. These studies collectively demonstrated that phenotypic plasticity of root and stomata could play a significant role in regulating the biomass productivity and stress-tolerant in cotton during drought.

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Introduction

Environmental stress such as drought severely affects crop productivity and survival across the Globe (Ullah *et al.*, 2018). Worldwide, the consequence of low and uncertain rainfall patterns causes drought that shrinks crop production and agricultural land (Ings *et al.*, 2013; Bertolino *et al.*, 2019). Stomata, the tiny mouth-like opening on the leaf epidermis, and their physiological control are important for plant adaptation and growth in the terrestrial ecosystem. These tiny pores are bounded by a pair of specialized cells, called guard cells. These cells play an essential role in stomatal functioning by integrating various endogenous and exogenous factors, which balance CO_2 uptake to water loss ratio between plants and their surrounding atmosphere (Blatt *et al.*, 2017; Watkins *et al.*, 2017). For decades, numerous studies have highlighted about stomatal regulation that is essential for plant growth and stress tolerance during drought, but the complete mechanism is still not yet explained (Yu *et al.*, 2016; Ullah *et al.*, 2017; Zhou *et al.*, 2017; Caine *et al.*, 2019).

Cotton is a multipurpose crop including lint-fiber, oilseed, and cattle feed, and grown in more than 80 countries in the world (Chen *et al.*, 2007; Jiang *et al.*, 2012). Numerous studies suggested, the growth and development of cotton plants are severely threatened by drought, which causes a loss of productivity and income (Wang *et al.*, 2017; Chen *et al.*, 2018). Therefore, we assumed that restricted water supply can balance water status and biomass accumulation in cotton plants by modulating the stomatal index and root growth.

Thus, in the presented work, we evaluated plant growth parameters, chlorophyll level and stomatal index in the two contrasting cotton varieties to water stress i.e., LRA-5166 (tolerant) and NBRI-67 (sensitive), grown under control and water stress conditions (Dubey *et al.,* 2022; Dubey *et al.*, 2023). To understand the variation in plant traits, such as stomatal features, root morphology regulates biomass accumulation were determined to monitor plant survival under drought stress. This manuscript will also provide deep insight into drought-responsive mechanisms for the development of plant phenome-based screening methods and breeding of drought-resilent cotton and other agricultural plant varieties.

Materials and methods

Plant materials, growth conditions, and drought treatment

Based on preliminary screening conducted in our laboratory, we have selected two contrasting varieties of cotton viz., LRA-5166 as drought tolerant variety, and NBRI-67 as a drought-sensitive variety for the present study (Dubey *et al.*, 2022; Dubey *et al.,* 2023). All experiments have been conducted in 5L plastic pots containing loamy soil (approx. pH of 7.7) obtained from CSIR-NBRI cropland, and mixed with 100g manure in each pot. Plants were grown in a polyhouse, at ambient agricultural environmental conditions (approx. 1000 μmol $m^{-2}s^{-2}$ sunlight, 40-42°C temperature, 37-40% relative humidity, and 415 ppm CO_2 concentration). 20 plants were used for each variety. After 54 days after sowing (DAS) all plants were randomly divided into two groups, half of the plants were subjected to daily watering (500 ml; well watered, control), and the other half were exposed to 15 days of water stress (100 ml water after one-day interval regularly).

Measurement of relative water content (RWC %)

In drought (during 15 days of water stress), differences in the rate of water loss were attributed to variation in leaf water status with a different time interval, for which RWC of the fully expanded leaves were investigated periodically (0, 5, 10 and 15th day of water stress). Fourth fully mature leaves were excised and immediately fresh weight (FW) was recorded, then the leaves were incubated in Milli-Q water (MQW) for 8h and then blotted for surface drying of the leaf. Subsequently, turgid weight (TW) was recorded, after that samples were kept in an oven at 70 °C for 72 h, and later dry weight (DW) was determined.

Relative water content was calculated as:

$$RWC (\%) = [(FW-DW)/ (TW-DW)] \times 100$$

Measurement of chlorophyll content (SPAD units)

Chlorophyll quantification was carried out for the two varieties of cotton grown in both water treatments using SPAD 502 (Osaka, Japan). The SPAD meter is described as a rapid and non-destructive method of chlorophyll quantification. The Fourth fully expanded leaves from the top were used for estimation of chlorophyll (Single Photon avalanche diode; SPAD unit). The middle portion of the leaf blade was used for this experiment.

Measurement of leaf morphology (Leaf area and petiole length)

Leaf area (LA; cm^2) was calculated by the leaf trace drawn on A4-size paper. For each treatment and each variety, 5 individual leaves (1 leaf/plant) were used and the leaf trace was drawn. The leaf traces were cut out and weighed (mg). For the estimation of the standard, a $1cm^2$ area of the same paper weight was recorded. Leaf area was calculated by the following formula:

LA (cm^2) =Weight of leaf trace/standard paper weight

Congruently same leaf petiole length was measured using a ruler.

Measurement of plant growth traits (root length, shoot length and biomass accumulation)

At the end of the 15th day of water stress, plants from each treatment were harvested. Roots were washed with running water to remove soil particles and spread on blotting paper for measurement of root and shoot length. The stem diameter was measured by the Vernier calliper. Then plants were cut into two parts, an above-ground portion (shoot i.e. leaf, stem, and branches) and a below-ground portion (root), and then fresh weight (g) was recorded. Subsequently, fresh plant samples were kept in an oven at 80 °C for 48 h to obtain root and shoot dry weight.

Light microscopic analysis of stomata

The analysis of stomatal morphology was conducted using the leaf impression method. Canada balsam was applied on the basal surface (abaxial surface) of leaves and imprints were developed. The leaf imprints were used for quantification of stomatal and epidermal cell numbers using an advanced bright-field microscope (Leica DM2500, Germany). Three random microscopic fields of view from the widest area of individual leaves were used. Two independent experiments have been conducted, 4 leaves were obtained from 4 different plants (i.e. 8 leaves the sum of two experiments) for each variety and each treatment. Stomatal index (SI;%) was calculated using the following formula; SI= 100×(number of stomata) / (number of stomata + number of epidermal cell and epidermal cell density (ECD) = number of epidermal cells/mm^2 of leaf area).

Statistical analysis

All graphs were prepared using the GraphPad Prsim5 software tool. The significant effects of water treatment on cotton varieties were analyzed by *t*-test using Microsoft Office Excel. The correlation between the different plant traits was analyzed using SPSS 16.0 software.

Results

Effect of water stress on relative water content (RWC), plant growth traits and chlorophyll content in cotton

Under water stress conditions, RWC significantly decreased in LRA-5166 by 36% and NBRI-67 by 40% (Fig. 1). On the other hand, there was no significant variation in chlorophyll content (SPAD unit) was observed in LRA-5166 and it was decreased in NBRI-67 compared to well water conditions in water stress (Table 1). LRA-5166 displayed less reduced leaf area and petiole length than NBRI-67 under water stress (Table 1). When plant growth attributes were analyzed to water stress conditions. Our data showed root growth was significantly higher in LRA-5166 than NBRI-67 under water stress compared to well water plants (Table 1). Although shoot length showed a significant reduction during water stress compared to well-watered plants of both varieties, NBRI-67 displayed more reduction than LRA-5166 (Table 1). The stem diameter decreased significantly in both varieties. However, the reduction in stem diameter in response to water stress was greater in NBRI-67 by 26% than in LRA-5166 by 15%, respectively (Table 1).

Table 1. Plant growth parameters of two contrasting cotton varieties LRA-5166 and NBRI-67 under well-watered and water-stress conditions

Parameters	LRA-5166		NBRI-67	
	WW	WS	WW	WS
Chlorophyll(SPAD unit)	34.2 ± 0.8	33.7 ± 0.5	37.4 ± 2.2	35.0 ± 1.0*
Leaf area (cm^2)	134.2 ± 11.6	92.5 ± 6.3***	131.8 ± 9.7	85.3 ± 3.2***
Petiole length (cm)	12.9 ± 1.5	7.0 ± 0.7***	14.0 ± 0.9	7.0 ± 0.7***
Root length (cm)	17.0 ± 1.5	18.5 ± 1.5	16.7 ± 1.2	13.5 ± 0.5*
Shoot length (cm)	128.0 ± 3.6	107.7 ± 4.9**	133.3 ± 4.2	89.0 ± 4.6***
Stem diameter (mm)	6.9 ± 0.5	5.9 ± 0.5**	7.9 ± 0.7	5.8 ± 0.4***
Root dry weight (g)	0.8 ± 0.02	1.1 ± 0.03***	1.4 ± 0.04	0.7 ± 0.07***
Shoot dry weight (g)	12.3 ± 2.3	11.3 ± 1.2	23.7 ± 1.5	8.0 ± 2.6***
Total dry weight(g)	13.1 ± 2.3	12.4 ± 1.2	25.0 ± 1.5	8.7 ± 2.7***
Root-shoot dry weight ratio	0.07 ± 0.01	0.09 ± 0.01*	0.06 ± 0.00	0.09 ± 0.02

Data are means ± SD. Asterisks represent significant changes between two water treatments: $*P < 0.05$;$**P < 0.01$;$***P < 0.001$.

Table 2. Correlation analysis of plant growth parameters of two contrasting cotton varieties i.e., LRA-5166 (tolerant) and NBRI-67 (sensitive) under well-watered and water-stress conditions

	SI	RL	SL	RDW	SDW	TDW	SPAD
SI	1	-0.276	0.559	-0.107	0.336	0.319	0.41
RL		1	0.569	0.552	0.312	0.324	-0.255
SL			1	0.639	0.798	0.795	0.431
RDW				1	0.894	0.902	0.625
SDW					1	1.000**	0.834
TDW						1	0.828
SPAD							1

Stomatal index; SI, plant growth features (root length; RL, shoot length; SL, root dry weight; RDW, shoot dry weight; SDW and total dry weight; TDW) with leaf chlorophyll content (SPAD unit) in cotton plants under well-watered and water stress conditions. Asterisks represent a significant correlation between all studied traits at $**p<0.01$ level.

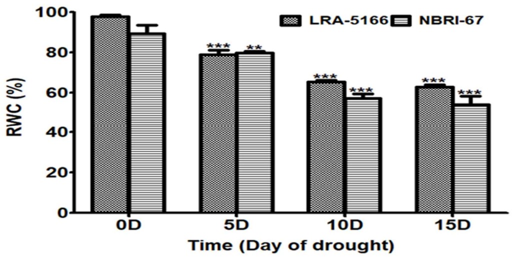

Fig. 1. Effect of water stress in relative water content (RWC;%) of two contrasting cotton varieties LRA-5166 and NBRI-67 plants. Data are means ± SD (n=4). Asterisks represent significant changes between two water treatments: $*P < 0.05$;$**P < 0.01$;$***P < 0.001$.

When effects of drought on root and shoot dry weight in two cotton varieties were analyzed. Water stress significantly increased root dry weight in LRA-5166, while it decreased significantly in NBRI-67 than well-watered plants (Table 1). When shoot dry weight was analyzed during water stress, there was unaltered shoot dry weight was observed in LRA-5166, while this treatment significantly decreased shoot dry weight in NBRI-67 than in well-watered conditions (Table 1). However, there was no significant variation observed between the two varieties during water stress. Likewise, during drought, the total dry weight of plants was unaffected in LRA-5166 and that was significantly affected in NBRI-67 than well-watered plants (Table 1). The root-shoot dry weight ratio was significantly increased in LRA-5166, while it was non-significantly increased in NBRI-67 under water stress.

Effect of water stress on stomatal density, stomatal index and epidermal cell density in cotton

Fig. 2 showed stomatal and epidermal cells (i.e. non-stomatal cells; pavement cells) responses during drought stress. Unaltered stomatal density was observed in LRA-5166, while it significantly increased in NBRI-67 during water stress than well-watered plants (Fig. 2A and Fig.S1A–D). However, when ECD was analyzed during drought, a significantly increased ECD was observed in each variety with higher values in LRA-5166 than NBRI-67 as compared to well-watered plants (Fig. 2B). Besides this, the decreased stomatal index was observed during water stress in both the varieties, but this was most affected in LRA-5166 than NBRI-67 compared to their corresponding well-watered plants (Fig. 2C).

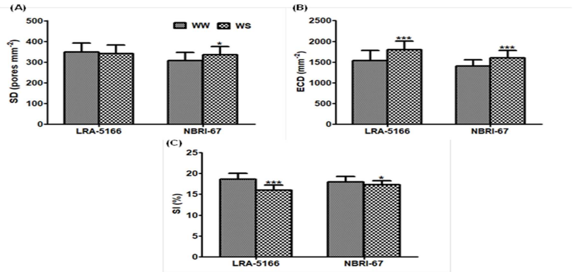

Fig. 2. Impact of water stress on stomatal density, index, and epidermal cell in two contrasting cotton varieties LRA-5166 and NBRI-67 leaves. The variation in (A) Stomatal density, (B) Epidermal cell density, and (C) Stomatal index. Values are means ± SD of two independent experiments (n=24). Asterisks represent significant changes between two water treatments: *$P < 0.05$;**$P < 0.01$;***$P < 0.001$.

The relationship between stomatal index and plant growth traits (RL, RDW, SL, SDW, and TDW) with chlorophyll content (SPAD units)

The Pearson correlation study (Table 2) showed that cotton leaf stomatal index was negatively related to RL and RDW, and was positively associated with SL, SDW, TDW and SPAD. Plant growth traits and chlorophyll content (SPAD units) were positively associated with each other, except root length with SPAD. Moreover, SDW was strongly correlated with TDW. This correlation analysis displayed reduced stomata with modifying root systems along with modulation of chlorophyll level contributing to the adjustment of plant growth and survival.

Discussion

Many studies have described that drought alters water status and stomatal characteristics in plants, which limits the growth and survival of plant species (Chen *et al.*, 2018; He *et al.*, 2018; Caine *et al.*, 2019). Here, we evaluated the impacts of water stress on water status, plant growth traits, and chlorophyll content as well as stomatal traits in two cotton varieties. Previously, phytochrome B (*phyB*) mutant analysis showed plants conserve water by regulating leaf area and stomatal numbers (Liu *et al.*, 2012). Our studies showed that water level significantly reduced in both the varieties of cotton, however, it was found less reduced in LRA-5166 than NBRI-67 during water limitation. Similar reduction patterns in leaf area and petiole length were observed in both varieties of cotton. These results suggested that plants modulate leaf architecture, which can potentially prevent water loss by reducing surface area during drought. There was no change observed for chlorophyll content in LRA-5166, however, NBRI-67 had reduced SPAD value in water stress. During drought, reduced chlorophyll content is associated with decreased plant biomass (Liu *et al.*, 2018), while increased chlorophyll content is considered an indicator of higher biomass accumulation and drought tolerance (Zhou *et al.*, 2017; Singh *et al.*, 2018). This demonstrates a greater ability to maintain biomass production (total dry weight) in LRA-5166 than NBRI-67 during drought.

To combat environmental stress, plants modulate stomatal number, which regulates biomass accumulation and abiotic stress tolerance (Meng and Yao, 2015; Hughes *et al.*, 2017; Caine *et al.*, 2019). Our results showed stomatal density was found unaltered in both the varieties of cotton under water stress. On the other hand, during water stress, both the varieties showed significantly increased epidermal cell density. When the stomatal index was analyzed in response to water stress, marked variation between LRA-5166 and NBRI-67 was observed. Based on this work, we demonstrated that the reduced stomatal index of both the varieties was caused by the increasing number of the epidermal cell compared to stomata on the leaf, as reported by Zheng *et al.* (2013).

Furthermore, a comparable increased number of pavement cells with reduced cell expansion was observed in both varieties during water stress. Collectively, these results reveal that the cotton plants modulate leaf area and stomatal index by controlling epidermal cell development during water stress. Furthermore, these results implied that reduced stomatal index and leaf development play crucial roles in the conservation of water in plants to drought stress.

Root length was found unaltered in LRA-5166 while it was found significantly reduced in NBRI-67 under water stress. By contrast, both the varieties displayed reduced shoot length and stem diameter, with less decrement in LRA-5166 than NBRI-67 during water shortage. When plant dry weight was analyzed during water stress, LRA-5166 exhibited higher root dry weight than NBRI-67. Previously, it was reported that higher root growth improves drought tolerance in plants (Meng and Yao, 2015; Silva *et al.*, 2019). Consistent with this, our study showed that higher root growth was attributed to variation in drought tolerance between LRA-5166 and NBRI-67. Although, shoot dry weight was observed unaffected in LRA-5166, while it decreased in variety NBRI-67 during water stress conditions. Similarly, total dry weight was found unaltered in LRA-5166, whereas it significantly decreased in NBRI-67. Interestingly, the root-shoot dry weight ratio was higher in LRA-5166, whereas it was found unaffected in NBRI-67 in response to severe water limitations. Earlier, it was reported that in drought response mechanism, plant regulates the production of stomata on leaf and root growth, which improves biomass and drought tolerance by adjusting water level to water stress (Meng and Yao, 2015; Yu *et al.*, 2016). The correlation study showed that stomatal index was negatively associated with root traits (root length and root dry weight), while positively related with total dry weight and chlorophyll. Therefore, we demonstrated that an integration of drought signal modifies the root system and decreases stomatal numbers along with the balanced chlorophyll level could modulate aerial biomass accumulation and enhance drought tolerance in cotton plants. We also suggested regulation of fine tuning between root and stomata play a predominant role in biomass accumulation than modulation of chlorophyll level in LRA-5166 than NBRI-67 during water stress conditions as previously reported by Meng and Yao (2015) and Hughes *et al.* (2017).

In conclusion, our findings explain that how cotton plants confer drought tolerance by evolving multiple features including regulation of chlorophyll molecule, enhanced root growth along with reduced leaf growth and stomatal index in response to water stress conditions. Thus, enlargement of the root is accompanied by decreased leaf area and stomatal index which maintain plant water relation (balance between uptake and loss of water, respectively) and biomass accumulation (total dry weight) during water deficit. Markedly, LRA-5166 showed less reduced RWC, balanced chlorophyll level, increased root growth and higher reduction in the stomatal index as well as more ability to modulate total dry weight than NBRI-67 during drought. Thus, LRA-5166 has been shown to be more drought tolerance than NBRI-67. However, the underline mechanism that involves the regulation of root and stomatal response in plants is still elusive so far and needs further molecular-physiological study.

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