Physiological, biochemical and molecular manifestations in response to seed priming with elicitors under drought in cotton

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Water stress has a detrimental effect on growth and development, which alters physio-biochemical activities. Seed priming with elicitors such as methyl jasmonate and paclobutrazol can mitigate the impact of drought stress. Therefore, pot-culture studies were conducted with drought-tolerant (DTS-155) and drought-susceptible (IC-357055) cotton genotypes to assess the seed priming effects of elicitors (methyl jasmonate and paclobutrazol) on the physio-biochemical changes and gene expression. The dose (50, 100, 150 and 200 mM) and time interval (1.5 and 2.5 h) experiments of both the elicitors were performed separately. On the basis of germination, seedling growth and vigour, a 150 mM elicitor for 1.5 h time interval was found to be the best. Biochemical and physiological parameters confirmed an increase in relative water content, total antioxidant activities, chlorophyll, superoxide dismutase, catalase and proline under drought stress in both the genotypes, but a decrease in lipid peroxidation. Among the elicitors, methyl jasmonate improved drought tolerance as compared to paclobutrazol. Gene expression studies with *Rub-S*, Rub-*L* and *Osmotin* confirmed the results. Transcript abundance of *Osmotin* and *Rub-L* was upregulated under drought stress in both the genotypes and was highest in methyl jasmonate primed samples. These findings suggest that priming with methyl jasmonate enhances drought tolerance in cotton.

Keywords: Drought responsive gene, *Gossypium hirsutum*, methyl jasmonate, paclobutrazol, seed priming.

COTTON (*Gossypium* spp.) is an important cash crop supporting the livelihood of millions of households[1]. Nearly half of the world's cotton acreage is rainfed, contributing only 27% to the total production[2]. The limited water availability in rainfed regions affects crop growth and yield. Drought is a major threat to plants, as deficit water alters the plant–water relations at molecular, cellular and organ levels to the whole plant[3,4].

A controlled hydration technique known as seed priming induces metabolism before germination, while discouraging radicle emergence[5]. It has effectively enhanced tolerance against various abiotic stresses like drought, salinity, chilling and heavy metals in different plant species[6]. Elicitors – the extrinsic or foreign molecules such as methyl jasmonate (MJ) elicit plant defence responses at low concentrations and are naturally produced in plants. It has been found that the exogenous application of MJ increases the antioxidant activity of plants under water stress[7]. Thus, we hypothesize that the use of elicitors may enhance the drought tolerance of cotton as well. Since little is known about the effect of elicitors on cotton plant growth, studies were conducted with the objectives (i) to standardize the dose of the elicitor for seed priming and (ii) to understand the physio-biochemical and molecular responses of seed priming with MJ and paclobutrazol priming (PB) on cotton genotypes under drought stress.

Materials and methods

Effect of dose of elicitors on cotton growth

Seeds of cotton genotypes DTS-155 (drought-tolerant) and IC-357055 (drought-susceptible) were separately treated with MJ and PB at four different concentrations, viz. 50, 100, 150 and 200 mM for different time intervals, viz. 1.5 and 2.5 h separately. After drying, the seeds were placed in a petri dish with germination paper to determine the germination percentage, seedling growth and vigour (fresh biomass). Seeds were sown in 36 pots (18 for each genotype) filled with equal amounts of soil and vermicompost and the experiment was designed in RBD (randomized block design) with six treatments. These were (i) no priming + no drought stress, (ii) no priming + drought stress, (iii) MJ priming + no drought stress, (iv) MJ priming + drought stress, (v) PB priming + no drought stress and (vi) PB priming + drought stress. Watering was done at regular intervals according to moisture levels in the soil. A 10-day drought stress was imposed twice, first at 50 days after sowing (DAS) and then at 80 DAS, as these two stages coincide with the floral bud and boll formation stages respectively. Leaf samples were collected and stored at –80°C for downstream processing.

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Relative water content

The relative water content (RWC) technique is widely accepted as a reproducible and meaningful index of plant water status[8]. A single leaf sample was taken and fresh weight was determined, followed by overnight flotation on water. After recording the turgid weight of the leaves, they were oven-dried to a constant weight at about 85°C. Observations were recorded at both stages and RWC was calculated using the following formula

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

where FW is fresh weight, DW the dry weight, TW is turgid weight.

Total chlorophyll content

A fresh leaf sample (0.25 g) was extracted with 10 ml acetone (80%). After filtration, the supernatant was collected and the volume was made up to 25 ml using acetone. It was mixed thoroughly and absorbance was noted at 663, 645 and 652 nm using a UV–Vis spectrophotometer (Shimadzu). The total chlorophyll content of fresh leaves was calculated as follows

$$\text{Total chlorophyll (mg g}^{-1}) = 20.2 \times \text{OD } 645 + 18.2 \times \text{OD } 633.$$

where OD is optical density.

Lipid peroxidation

The extent of lipid peroxidation was quantified in terms of TBARS content[9]. The leaf sample (0.5 g) was homogenized in 10 ml 0.1% trichloroacetic acid (TCA) and then centrifuged at 15,000 *g* for 15 min. Next, 4.0 ml of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA was added to 1.0 ml aliquot of the supernatant. After heating the mixture at 95°C for 30 min, it was cooled down in an ice bath, which was then centrifuged at 10,000 *g* for 10 min, the absorbance of the supernatant was recorded at 532 and 600 nm. The TBARS content was calculated in accordance with its extinction coefficient of 155 mM^{-1} cm^{-1}.

Proline content

Proline content of the leaves was estimated following the standard method of Bates *et al.*[10], with some modifications. First, 0.5 g leaf tissue was crushed in 10 ml of aqueous 3% sulphosalicylic acid. The filtered homogenate was mixed with acid ninhydrin and glacial acetic acid in equal amounts (2 ml). After heating the mixture for 1 h at 100°C, the reaction was terminated in an ice bath. To this, 4 ml toluene was added and vortexed for 10–15 sec. The chromophore-containing toluene layer was aspirated-off from the aqueous phase and kept at room temperature. Absorbance was read at 520 nm using toluene as a blank.

Catalase activity assay

The standard method of Chance and Maehly[11] was used to measure the catalase activity (CAT) of cotton samples. First, 5 ml of ice-cold buffer containing 50 mM potassium phosphate buffer (pH 7) and 1% (w/v) polyvinylpyrrolidone was used crushing 1 g of fresh leaf tissue. For CAT assay, a reaction mixture (3 ml) containing 100 mM Na_2HPO_4 buffer, pH 6.8 (2 ml), 30 mM H_2O_2 (0.5 ml) and 0.5 ml enzyme was prepared for each sample. CAT activity was assessed by noting the consumption of H_2O_2 (extinction coefficient, 39.4 mm^{-1} cm^{-1}) at 240 nm over a 3 min interval.

Superoxide dismutase

Superoxide dismutase (SOD) activity was determined using NBT and riboflavin method[12]. The protein was estimated employing Bradford's[13] method using BSA as the standard. Resolving and stacking gel for native-PAGE, i.e. without SDS and a denaturing agent, was poured and allowed to polymerize. The gel was then loaded with samples prepared in a 10× loading buffer. After completion of the running procedure, the staining step was executed first with buffer and then with riboflavin and NBT. Then gel was placed on a glass plate and exposed to a white light box until staining was sufficient.

Expression analysis of selected genes using quantitative real-time PCR

Total RNA was extracted from the leaves at both stages. Quantity and quality were analysed with a Qubit assay kit (Promega), RNA integrity was confirmed on 1.2% agarose gel and cDNA was prepared using a reverse transcriptase cDNA synthesis kit. Gene-specific primers were designed using Primer-3 software. The expression of drought-responsive and key photosynthetic genes (*Osmotin*, *Rubisco-S* and *Rubisco-L*) were analysed in DTS-155 and IC-357055 by quantitative real-time PCR (qRT-PCR). The 2× SYBR green mix (Promega) was used as the reaction mix to run the cycle on real-time PCR. The thermal profile was 10 min at 95°C, followed by 40 cycles each at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, followed by 1 cycle each at 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec for the dissociation curve. The assay was performed in duplicate (Table 1).

Statistical analysis

Randomized block design (RBD) used for statistical analysis had two genotypes, six treatments and three replications. The data were analysed using MS-Excel and Op-stat software (http://14.139.232.166/opstat/). Variation among treatment means was compared using the least significant difference ($P \leq 0.05$).

Results

Seed priming increases relative water content

Genotypic variation for RWC was not visible, though DTS-155 had more RWC (0.7% and 12%; non-significant) than IC-357055 (0.7% and 14%; significant) (Figure 1).

Table 1. Primer sequences for gene expression analysis

Primer ID	Primer sequences (5′–3′)	Length (nt)
Rubisco-S	F: TAACATGGTCGCTCCTTTCAC	21
	R: GGCCACACCTAGAAAACAACA	21
Rubisco-L	F: AAGATCGAAGCCGTGGTATTT	21
	R: AAAGTTCCTCCACCGAATTGT	21
Osmotin	F: GCTATGAAATCCGCAATGAGT	21
	R: TTGAAGGAGTCCACCACAATC	21
Actin	F: TACAACGAACTCCGTGTTGCTCCT	24
	R: TCACCGGAATCCAGCACAATACCT	24

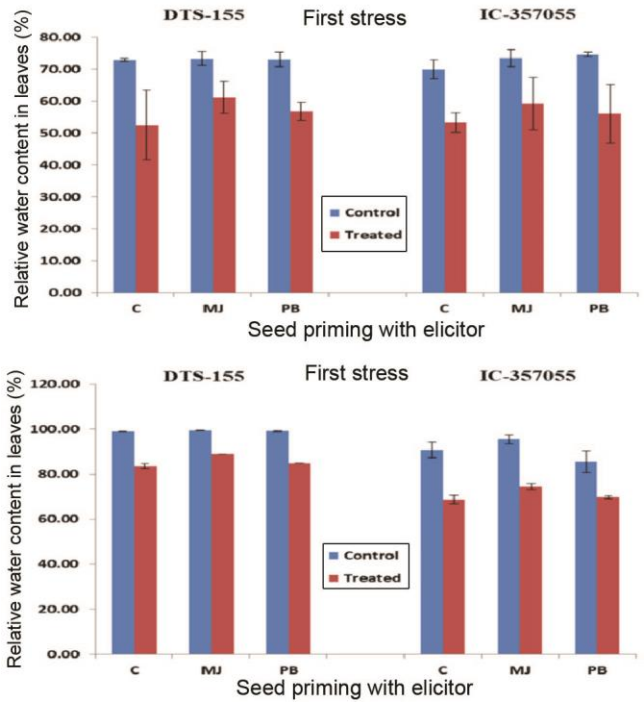

Figure 1. Effect of seed priming on relative water content in the leaves of cotton genotypes DTS-155 (drought-tolerant) and IC-357055 (drought-susceptible) under control and drought stress conditions at two different stages. *Bar represents the standard error. C, Unprimed; MJ, Methyl jasmonate priming; PB, Paclobutrazol priming.

However, under drought stress, RWC was found to decrease. In the case of MJ-primed samples, a decrease in RWC was minimum at both the stages (17% and 16%), whereas unprimed samples were found to have more loss in RWC at both the stages (27% and 19% respectively) (Figure 1 and Supplementary Table 1).

Effect of seed priming on total chlorophyll content

Total chlorophyll content was found to increase in the first and second stages in the DTS-155 genotype (7% and 13% respectively) under drought stress, whereas in IC-357055, it was found to decrease by 8% and 15% at both the stages respectively (Figure 2 and Supplementary Table 2). It was also observed that seed priming with elicitors lowered stress-induced chlorophyll degradation in the leaves of both genotypes. Minimum chlorophyll degradation (10%) was found in MJ-primed samples and maximum chlorophyll degradation was found in unprimed samples at both stages (31% and 27% respectively).

Elicitors lower lipid peroxidation in leaves under drought stress

Lipid peroxidation in terms of MDA level was found to decrease (56% at first stress and 30% at second stress) in DTS-155 under drought stress, while it was found to increase at both the stages (35% and 23% respectively) in the IC-357055 genotype (Figure 2, Supplementary Table 2). Seed priming lowered lipid peroxidation in the leaves of both genotypes under stress. Increase in lipid peroxidation under drought stress due to seed priming with MJ was less, i.e. up to (25%) at both the stages, whereas PB-primed and unprimed samples showed maximum lipid peroxidation (42% and 44% respectively).

Seed priming enhances proline content under stress

Proline content was found to increase under drought in the first and second stages in DTS-155 (28% and 25% respectively). On the other hand, it decreased at both stages (39% and 34% respectively) in the susceptible IC-357055 genotype (Figure 3 and Supplementary Table 3). Water stress enhanced the accumulation of proline, wherein all the treatments showed significant differences. A maximum increase was found in MJ-primed samples, followed by unprimed samples at both stages (90% and 91%). An increase in proline content was found to be the lowest (87%) in PB-primed samples.

Seed priming increases catalase activity under drought stress

Under drought stress, CAT was found to increase in DTS-155 by 66% at the first stage and 34% at the second stage,

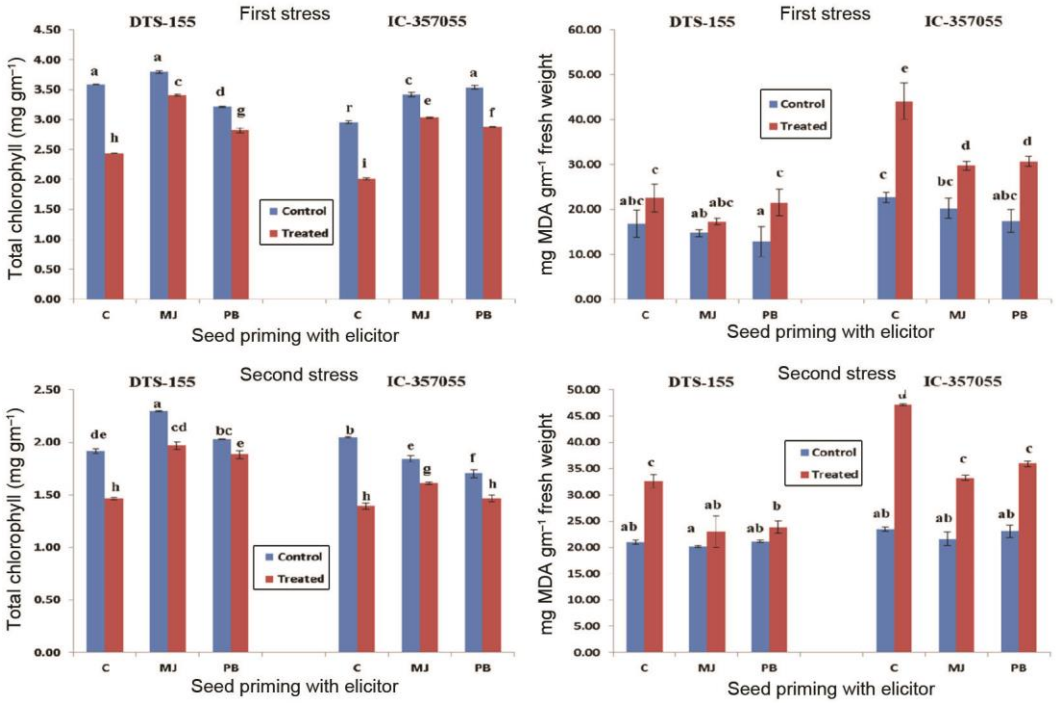

Figure 2. Effect of seed priming on total chlorophyll content and level of lipid peroxidation of cotton genotypes DTS-155 and IC-357055 under control and drought stress conditions at two different stages. *Columns with different lowercase letters indicate significant difference at $P < 0.05$ (Duncan's multiple range test).

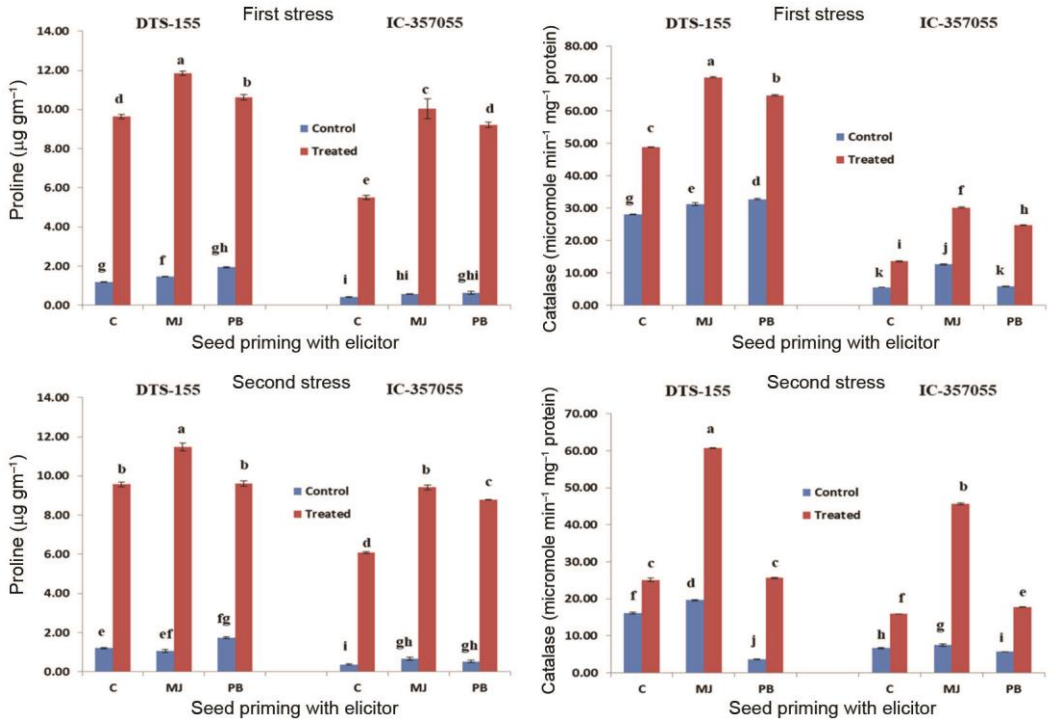

Figure 3. Effect of seed priming on proline content and catalase activity of cotton genotypes DTS-155 and IC-357055 under control and drought stress conditions at two different stages.

while it was found to decrease in IC-357055 by 198% and 52% at both stages respectively (Figure 3 and Supplementary Table 3). Moreover, seed priming increased CAT and a significant difference was found among all the treatments. Maximum CAT was found in PB-primed samples at both stages, followed by MJ-primed samples. Unprimed samples

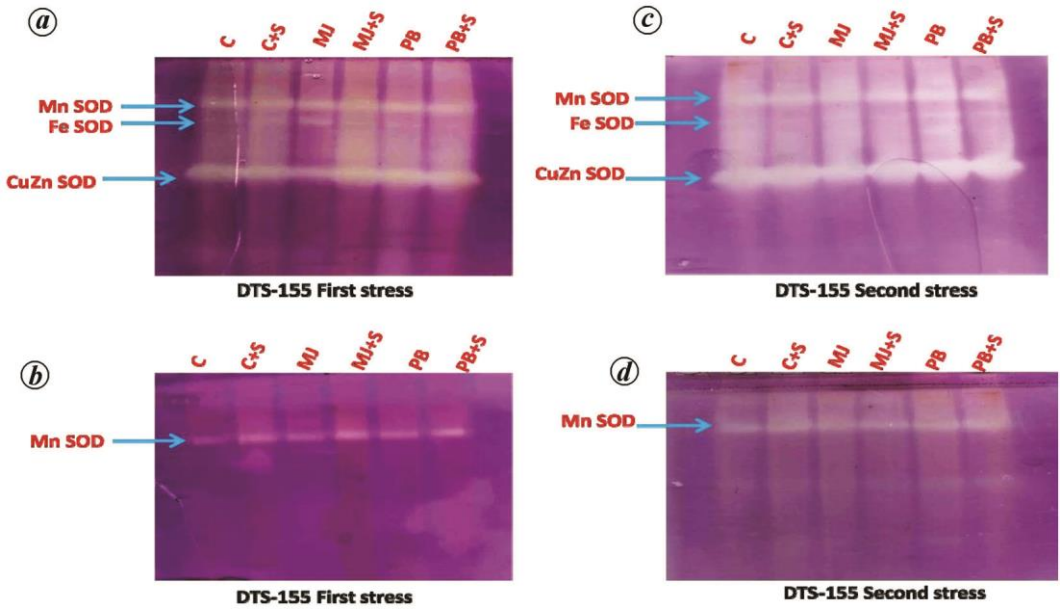

Figure 4. ***a***, ***c***, Effect of seed priming and drought stress on gel SOD activity in cotton genotype DTS-155 at two different stages. ***b***, ***d***, H_2O_2 sensitivity test for SOD isoforms.

showed a minimum increase in CAT (46% in the first stage and 44% in the second stage).

Seed priming increases superoxide dismutase activity under drought stress

Superoxide dismutase (SOD) activity was found to increase under drought stress in all the treatments, but it was highest for both genotypes in the MJ + S-primed treatment at both the stages of stress. We could also observe genotypic variation for SOD activity as well as SOD isoforms, wherein Fe-isoform was found to be more expressive in DTS-155 (Figure 4) compared to IC-357055 (Figure 5). Further, we tested SOD for H_2O_2 sensitivity results, wherein the Fe–SOD and Cu–Zn–SOD isoforms were missing in all the treatments (Figures 4 and 5).

Expression profiling of key photosynthetic and stress-responsive genes using qRT-PCR

The transcript level of different genes associated with drought stress as well as photosynthesis was measured using qRT-PCR. Among stress-associated genes, expression profiling of *Osmotin*, *Rubisco*-large subunit (*Rubisco-L*) and *Rubisco*-small subunit (*Rubisco-S*) was done. The relative expression of these genes was found to be upregulated under drought stress. *Osmotin* showed a maximum change in MJ primed samples, i.e. 10.63-fold in DTS-155 followed by PB-primed samples (8.37-fold) and 4.5-fold in IC-357055 followed by PB-primed samples under stress in the first stage (Figure 6). In the second stage of stress, maximum change was found in MJ-primed samples (6.89-fold) in DTS-155, followed by MJ-primed samples in IC-357055 (2.01-fold) (Figure 6). The expression level of *Rub-L* was found to be maximum in MJ-primed samples (4.67-fold) in DTS-155, followed by MJ-primed samples (2.30-fold) in IC-357055 in the first stage of stress (Figure 7). During the second stage, maximum expression was found in MJ-primed samples (4.18-fold) in DTS-155 followed by MJ-primed samples (2.6-fold) in IC-357055 (Figure 7). Further, expression of *Rub-S* also revealed maximum fold changes in MJ-primed samples (1.63-fold) in DTS-155, followed by MJ-primed samples (1.31-fold) in IC-357055 in the first stage of stress. At the second stage of stress, MJ-primed samples (1.35-fold) were found to have maximum fold expression in IC-357055, followed by unprimed, stress-treated samples (1.04-fold) in DTS-155 (Figure 8). Semiquantitative RT-PCR showed a marked difference in the band intensity and band pattern of *Osmotin*, *Rub-L* and *Rub-S* genes compared to *actin* gene when run on 2% agarose gel. The abundance of *Osmotin* and *Rub-L* was more in stress-treated samples (C + S, MJ + S and PB + S) compared to the respective controls (C, MJ and PB), whereas the transcript level of *Rub-S* decreased under stress in most cases. Among all the treatments, the MJ + S treatment showed higher band intensity in both genotypes at both stages of stress (Figures 9 and 10).

Discussion

Cotton metabolism and yield have always been compromised under water-deficit conditions. The reduced rainfall with regularly changing patterns are causing frequent onset of droughts around the world[14]. Our findings indicate

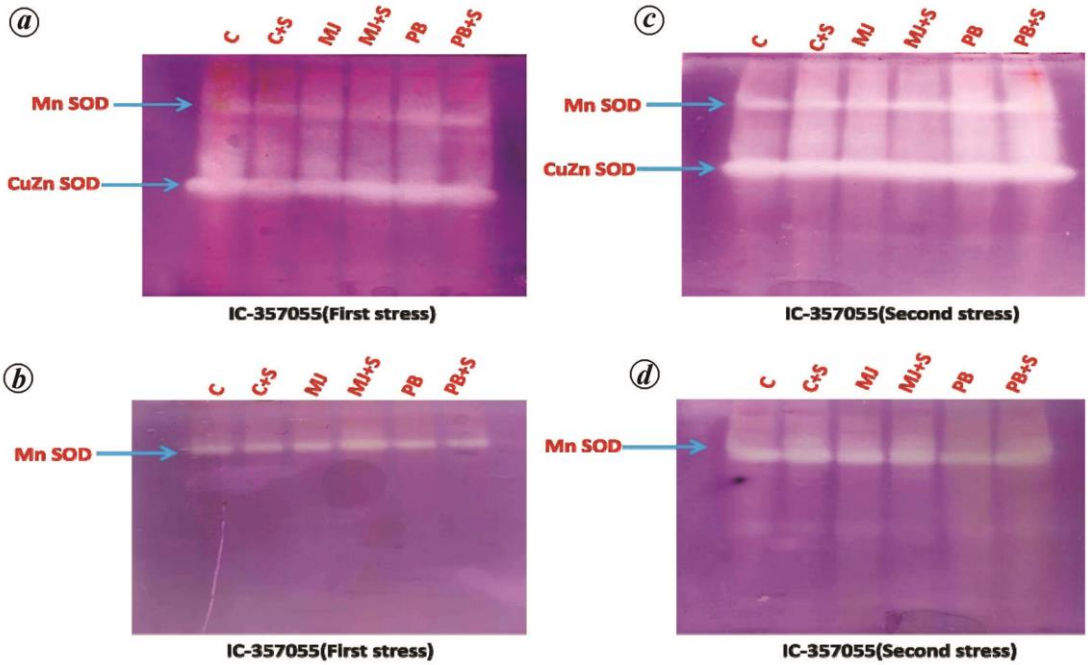

Figure 5. ***a***, ***c***, Effect of seed priming and drought stress on gel SOD activity in cotton genotype IC-357055 at two different stages. ***b***, ***d***, H_2O_2 sensitivity test for SOD isoforms.

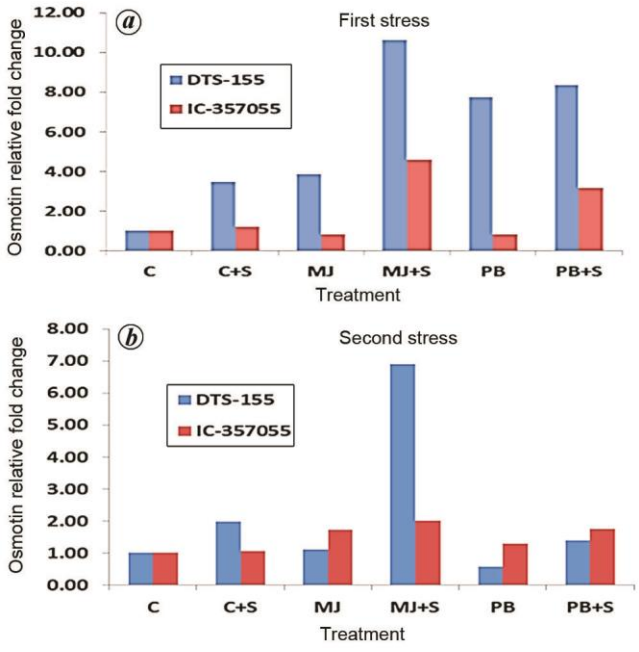

Figure 6. Quantitative real-time expression profiling of *osmotin* in different genotypes of cotton under drought stress at different stages (***a***, first stress and ***b***, second stress). Actin was used as the endogenous control gene. Relative fold expression was calculated using the method of Pfaffl[28].

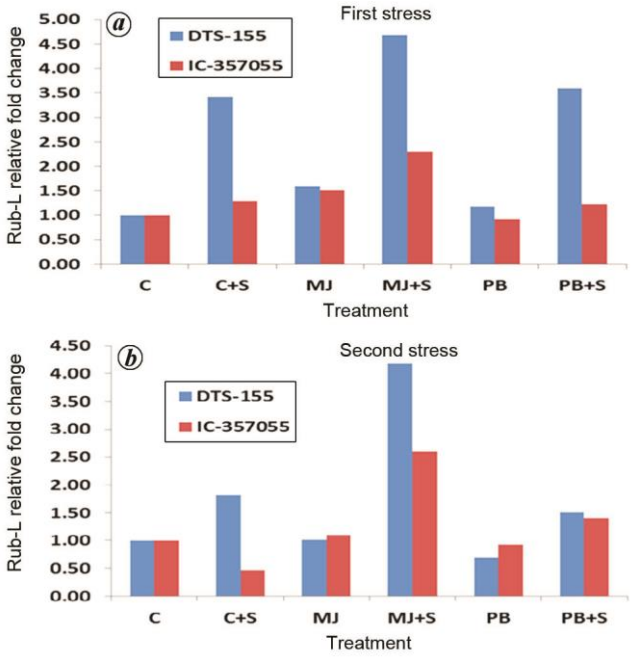

Figure 7. Quantitative real-time expression profiling of *Rub-L* in different genotypes of cotton under drought stress at different stages (***a***, first stress and ***b***, second stress).

that drought tolerant genotype DTS-155 maintained a better level of RWC under water stress condition than the susceptible IC-357055. Seed priming with elicitors could significantly improve RWC (17% and 16% decrease in primed samples at both stages) under stress compared to unprimed seeds. Cotton responds to drought stress physiologically through stomatal closing, root development, altered photosynthesis rate and other cellular adaptations[15]. With a decrease in irrigation water, chlorophyll content in the leaves of *Rosmarinus officinalis* showed a decline, which was

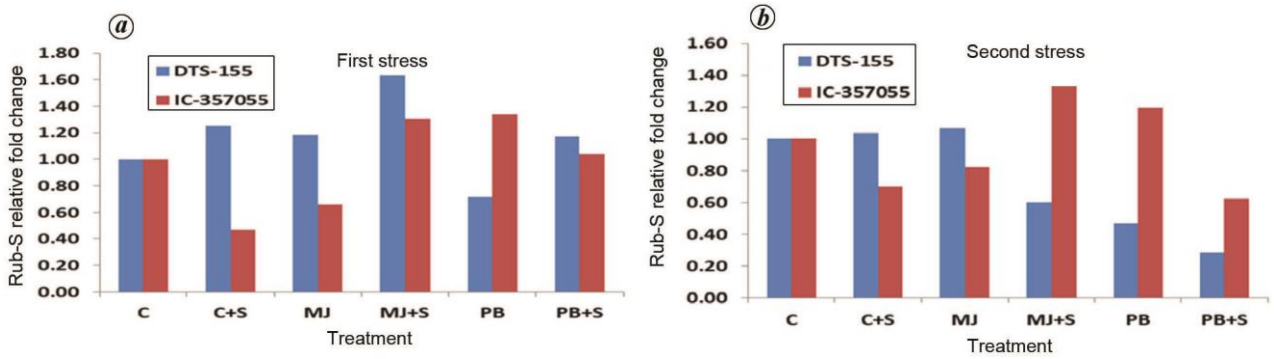

Figure 8. Quantitative real-time expression profiling of *Rub-S* in different genotypes of cotton under drought stress at different stages (***a***, first stress and ***b***, second stress).

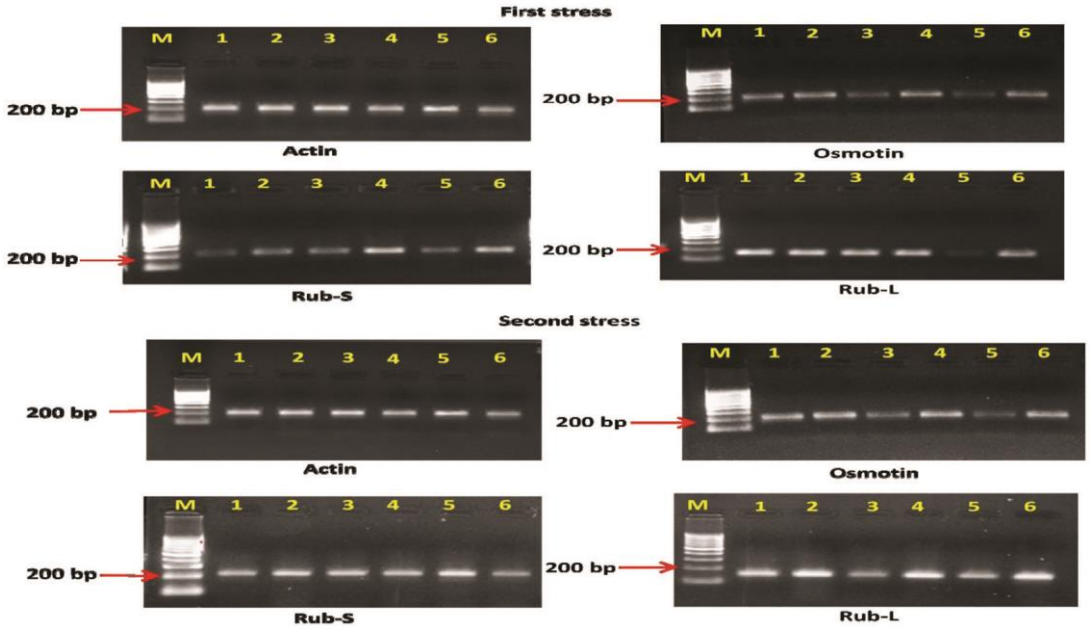

Figure 9. Effect of seed priming and drought stress on gene expression in cotton genotype DTS-155 at both stages of stress. M, Marker (100 bp), lane 1, C; lane 2, C + S; lane 3, MJ; lane 4, MJ + S; lane 5, PB; lane 6, PB + S. C, Control (no elicitor treatment + no drought stress); S, stress; MJ, Methyl jasmonate; PB, paclobutrazol; MJ + S, MJ with stress; PB + S, PB with stress.

further found to be correlated with RWC[16]. Our results also suggest that chlorophyll content decreases after a dry spell.

Alteration in the fluidity status of the cell membrane as stress perception is a good indicator for measuring stress level in terms of MDA[17]. As the dry spell/drought progresses, the levels of lipid peroxidation increase in the leaves by two- to fourfold with a positive correlation with protein peroxidation[18]. In this study, lipid peroxidation increased under drought stress, though priming with elicitors could check lipid peroxidation to a great extent (56% less increase at first stress and 30% at second stress in DTS-155). Following surface and membrane alterations, the cell has to deal with internal perturbations as well. Osmotic adjustment is one such adaptation, which reduces the effects of drought-induced damage in crop plants. Of many osmolytes or solutes, proline is the most studied in stress tolerance. Our results indicate more proline accumulation under stress conditions. However, plants raised from seed priming with elicitors, especially MJ, showed a significant increase in proline content (28% at first stress and 25% at second stress in DTS-155) under drought. It has been shown that free proline accumulates up to 100 times the concentration of well-watered controls as the stress increases[19].

Induction and production of reactive oxygen species (ROS) under stress are inevitable, but the activity of antioxidant enzymes such as catalase and SOD significantly increases to scavenge ROS until the plant recovers from stress, as reported in cotton[20]. In numerous plant species, such as *Allium* and Kentucky grass, higher CAT conferred

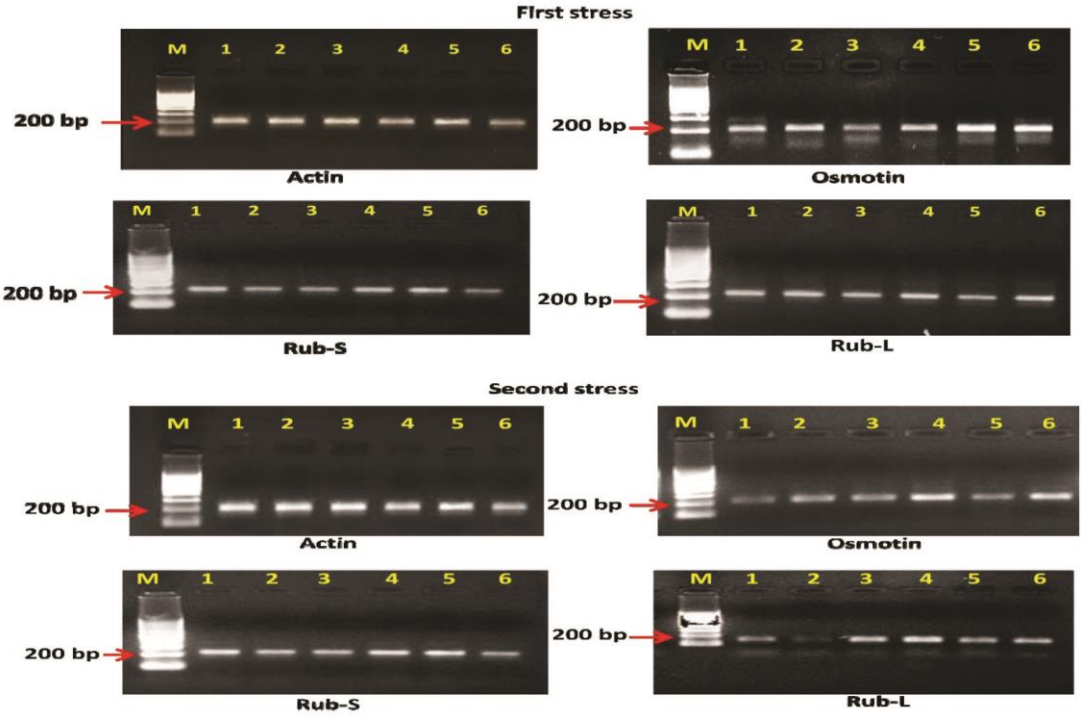

Figure 10. Effect of seed priming and drought stress on gene expression in cotton genotype IC-357055 at both stages of stress. M, Marker (100 bp); lane 1, C; lane 2, C + S; lane 3, MJ; lane 4, MJ + S; lane 5, PB; lane 6, PB + S.

tolerance to water scarcity by nullifying the effect of H_2O_2, thereby protecting the plants against oxidative stress[21,22]. In our findings, CAT was enhanced under drought stress, which was even more for seed priming with elicitors done (66% and 34% increase in DTS-155). SOD is another key component of the cell protection system against oxidative stress. Our results suggest that in chloroplasts and mitochondria, Fe–SOD and Mn–SOD may play a crucial role in the detoxification of superoxide radicals. Similar findings have been reported in wheat[23] and *Sesamum indicum*[24].

Molecular responses to stress include either the induction or suppression of stress-responsive genes. Since photosynthesis is a primary metabolic pathway which is affected by the perception of any stress, leading to disturbed osmotic status of the plant cells, we analysed the expression of key photosynthetic enzymes, i.e. Rubisco large and small subunits, alongwith osmotins for their differential expression studies. Osmotins are considered to be induced against several biotic and abiotic challenges in diverse plant species[25]. Osmotin and Rubisco activase are also found to increase in response to ABA treatment and drought or salt stress induced by polyethylene glycol-mediated[25–27]. In the present study, a similar expression pattern of *osmotin* and *Rub-L* was observed, whereas expression of *Rub-S* declined under drought stress, which is justified because of the presence of tight binding inhibitors for the same. Priming with MJ and PB increased the *osmotin*, *Rub-L*, *Rub-S* fold expression in tolerant genotype followed by susceptible genotype.

Conclusion

In this study, between two elicitors used for priming, MJ priming for 1.5 h at 150 mM concentration showed better efficiency over PB in inducing the physio-biochemical and molecular manifestations for improving drought tolerance in cotton genotypes.

Conflict of interest: The authors declare they have no conflict of interest.

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