Dissection of phenylpropanoid pathway during salt stress in cotton (Gossypium hirsutum L.)

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DOI: https://doi.org/10.22271/chemi.2020.v8.i1a.8303

Abstract
Salt stress is an abiotic stress in cotton (G. hirsutum L.) that affects evenly yield. The present investigation carried out to study the significance of integrative biochemical and molecular approaches against salt stress in cotton. Four different varieties, two salt tolerant (G.Cot.-16, GISV.218) and two susceptible (G. Cot.10, G.Cot.-100) were used screened at different salinity levels viz., 0.8 dS/m, 3 dS/m, 7 dS/m and 10 dS/m. Bio-chemical and molecular parameters analysed at seedling (15 DAS) and squaring stage (45 DAS). Biochemical traits like Protein content, total phenol content and Proline content was found to be increased with increasing salinity levels. Enzyme activity related to phenylpropanoid pathway (PAL, C4H and C3H) was found to be also increased with the advancement in salt stress, hence it implies possible role of these enzymes in imparting tolerance to salt stress in cotton. Reactive oxygen species (ROS) scavenging enzymes such as SOD, POD, CAT and PPO recorded increase in their activity at both seedling and squaring stages with increasing salinity levels. Expression analysis of key genes of phenylpropanoid pathway via PAL, C4H (C4H1 and C4H2) revealed maximum fold expression of PAL and C4H1 in the leaves, whereas least fold expression was observed for C4H2. Thus, it may be concluded that phenylpropanoid pathway play significant role in imparting tolerance against salt stress in cotton.

Keywords: Cotton, phenylpropanoid pathway, PAL, C4H

Introduction
Cotton, the white gold, is the world’s leading fibre crop and second most important oilseed crop. Man has utilized cotton for his benefits since ancient times (Fryxell, 1992). Salinity is prior threat to world agriculture and it imposes a major setback in increasing the yield of cotton. Salt stress suppresses the growth in general across the plants but the rate of reduction in growth only depends on the tolerance level of plants species. Generally, salt stress reduces the water potential in the soil and causes disturbances in ion-homeostasis and ion toxicity in plant cells that repress enzymatic activity (Zhang, et al., 2001) [16]. Plant growth suppression is directly related to the total concentration of soluble salts or the osmotic potential of soil water (Greenway & Munns, 1980) [13]. Salt damage of leaf tissues is usually the result of Na+ accumulation in leaf cells that shortens the life span of individual leaves, thus reducing their net photosynthetic productivity and crop yield. Increased NaCl levels result in a significant decrease in the root, shoot, leaf biomass, increase in the root/shoot ratio, and cottonseed abortion leading to both yield loss and lower fibre quality (Davidonis, et al., 2005) [5]. Generally salt tolerance in cotton has been associated with Na+ exclusion. High salinity reduces N and P uptake in cotton, whereas low salinity does not have a significant effect on the absorption of either of the ions. (Yaser et al., 2018) [39] Phenylpropanoid compounds are precursors to a wide range of phenolic compounds that contributes plant responses towards biotic and abiotic stimuli. They are not only indicators of plant stress responses but are also key mediators of the plant’s resistance/tolerance towards pests, mechanical or environmental damage, like salinity, drought, or wounding (Camera, et al., 2004) [20]. With the increase of salinity stress, most plants become unable to extract water from the soil and face osmotic stress. Soil salinity also disturbs nutrient balance in soil as well as in plants and creates specific ion toxicity in plant. (Muhammad et al., 2018) [27] Therefore, the present work aims study the effect of salinity levels on biochemical parameters and molecular responses especially enzymes genes of phenylpropanoid pathway.
Materials and Methods
The experiment was conducted at, Main Cotton Research Station, Navsari Agricultural University, Surat, Gujarat. The seed of four cotton varieties of G. Cot-16 (Tolerant), GISV 218 (Tolerant) G. Cot 10 (Susceptible) and G. Cot 100 (Susceptible) were used for experiment. In pre-constructed channels, different levels of salinity viz., 0.8 dS/m, 3 dS/m and 10 dS/m. were maintained with four plants per treatment per replication in complete randomised design with factorial concept (FCRD).

Biochemical parameters
Fresh second upper leaf samples were collected and washed twice with tap water followed by deionized water. All biochemical parameters were analyzed at 15 and 45 day after sowing (DAS).

Proline was estimated by using acid ninhydrin method as described by Bates, et al., (1973) [3]. Phenol extraction and estimation were elucidated by the method as described by Malick & Singh, (1980) [24]. Peroxidase (POD) activity was estimated as described by Reddy & Gasber, (1971) [32]. Superoxide dismutase (SOD) activity was measured by the method as described by Beyer & Fridovich, (1987) [4]. Catalase (CAT) and polyphenol oxidase (PPO) activity was measured by method as described by Thimmaiah, (1999) [33]. Na+ and K+ ratio from root and shoot was estimated by flame photometer as described by AOAC, (1990) [1]. Enzyme activity related to phenylpropanoid pathway viz. Phenylalanine ammonia lyase (PAL) activity was measured by the method as described by Mahatma, et al., (2011) [23]. Cinnamic acid-4-hydroxylase (C4H) and p-Coumaric acid-3-hydroxylase (C3H) activity was measured by the method as described by Padhiar & Sharma, (2008) [30].

Molecular analysis
RNA was extracted from Fresh leaves (0.1 g) collected at 15 and 45 days after sowing (DAS) from treated and non-treated plants of each genotype and were powdered in liquid nitrogen and 45 days after sowing (DAS) from treated and non treated plants of each genotype and were powdered in liquid nitrogen. RNA was extracted using Spectrum™ Plant Total RNA Kit (Sigma).

Synthesis of cDNA
The cDNA was synthesized by using high capacity cDNA reverse transcription kit (thermo) following manufacturer’s instructions. For cDNA synthesis, 2µl of template RNA (100 ng), 1µl of Oligo (dT) primer (0.5g) and 8µl of nuclease free water were mixed by brief centrifugation and incubated at 65°C for 5 min followed by immediate cooling on ice. After that 4 µl of 5x reaction buffer, 1 µl of RibolockRNase Inhibitor (20 u/µl), 2 µl of 10 mM dNTP mix and 2µl of M-MuLV Reverse Transcriptase (20u/µl) were mixed by brief centrifugation and incubated for 60 min at 37°C. The reaction was terminated by heating at 70 °C for 5 min. The reverse transcription reaction products (cDNA) were stored at -70 °C until used.

Gene specific primers were used to amplify a set of cotton pphenylpropanoid pathway key genes and housekeeping genes in quantitative real time-PCR (qRT-PCR) assays and details are given in Table 1. Gene specific primers PAL, C4H, C4H2 & Ubiquitin were selected according to the Xu et al. (2011).

Table 1: Sequences of primers used for expression analysis

| Primer | Sequence | Gene ID |
|--------|----------|---------|
| PAL F  | CAATGGACAATGCTGTGTTGCTAT | CL1Contig4241 |
| PAL R  |CACGGTGTCCTCTCATGTGCTTCTT  | CL23Contig4  |
| C4H1 F | ATCCAGAGATCCAGCAAAATTGG  | CL23Contig5 |
| C4H1 R | GACACGCTCTTCTCTCAACACAA  | CL23Contig5 |
| C4H2 F | GGGATCTCACTGTAGGTTGCGGTGG  | DQ116441 |
| C4H2 R |GGCTAAAGTCGTAGGTTGCGGTGG  |  |
| Ubiquitin F | ACCAAAAATCCAAAAGATAGGAGAAGACAT |  |
| Ubiquitin R |ACGCAAACCGAGCAGAAGTTGAGAG  |  |

Results and Discussion
Biochemical parameters
Biochemical analysis was done from fresh sample that was collected from cotton plant at seedling (15 DAS) and squaring stage (45 DAS). Sample collected from normal plot was served as control.

Significant varietal differences in response to salinity had been observed. Maximum proline content at 15 DAS was observed at highest salinity level i.e.10 dS/m in G.Cot.-16 (0.86 mg/g tissue) followed by GISV-218 (0.77mg/g tissue), G. Cot-10 (0.45 mg/g tissue) while, it was found minimum in susceptible variety G. Cot 100 (0.33mg/g tissue) as compared with control. Further, at 45 DAS it was observed that at highest salt concentration proline content was significantly increased in tolerant genotype i.e. GISV-218 (1.35 mg/g tissue) followed by G.Cot-16 (1.30 mg/g tissue) while, it was found minimum in susceptible genotype G.Cot-100 (0.74mg/g tissue) followed by G. Cot 10 (0.97 mg/g tissue). However, proline content was increased with increase of salinity level in the genotype studied. (Figure 1)

![Fig 1: Change in Proline content in leaves of cotton during different level of salt stress](image-url)
At highest salinity (10 dS/m) there was significant difference recorded in total phenol content among the varieties. At the seedling stage total phenol content was found on par in resistant variety GISV-218 (18.86 mg/g tissue) and G. Cot.-16 (18 mg/g tissue) followed by G.Cot.100 (12.40 mg/g tissue). While, least phenol content was found in G.Cot.-10 (11.60 mg/g tissue). Similarly at squaring stage also it was found at par in resistant variety GISV-218 (21.26 mg/g tissue) and G.Cot-16 (20.49 mg/g tissue) followed by G.Cot.100 (14.55 mg/g tissue) While, least phenol content was found in G.Cot.-10 (14.11 mg/g tissue). From these results it can be concluded that salinity significantly affects total phenol content at both stages. (Figure 2)

![Total Phenol content (mg/g of tissue)](image1)

**Fig 2:** Change in Total Phenol content in leaves of cotton during different level of salt stress

At 15 DAS peroxidase (POD) was highest in G.Cot.-16 (7.81 unit/mg protein) followed by GISV-218 (8.61 unit/mg protein) while it was lowest in G.Cot.-100 (4.89 unit/mg protein) followed by G.Cot.-10 (4.91 unit/mg protein) under most salty condition (10 dS/m). As compare to control it was 4.3, 4.2, 5.7 and 6.8 fold higher in G.Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively. At 45 DAS POD activity was maximum in GISV-218 (8.82 unit/mg protein) followed by G.Cot.-16 (8.10 unit/mg protein) while it was lowest in G.Cot.-100 (5.35 unit/mg protein) followed by G.Cot.-10 (5.49 unit/mg protein) under high salinity condition (10 dS/m). As compare to control it was 4.42, 4.10, 5.53 and 6.61 fold increase in G.Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively. (Figure 3)

![POD specific Activity (mg/g of tissue)](image2)

**Fig 3:** Change in peroxidase (POD) specific activity in cotton leaves during different levels of salt stress

At both the stages SOD enzyme activity was observed higher in tolerant genotype then in susceptible genotype. At 15 DAS it was highest in GISV 218 (0.33 unit/mg protein) followed by G.Cot-16 (0.30 unit/mg protein) while it was lowest in G.Cot.-100 (0.19 unit/mg protein) followed by G.Cot.-10 (0.21 unit/mg protein) under highest saline condition (10 dS/m). Similar trend was found at 45 DAS, the SOD activity was maximum in GISV-218 (0.35 unit/mg protein) followed by G.Cot.-16 (0.34 unit/mg protein) while it was lowest in G.Cot.-100 (0.23 unit/mg protein) followed by G.Cot.-10 (0.25 unit/mg protein). SOD activity was enhanced at both stages with increasing salinity levels. (Figure 4)
At 15 DAS Catalase enzyme activity was highest in GISV-218 (0.123 unit/mg protein) followed by G.Cot.-16 (0.119 unit/mg protein) while it was lowest in G.Cot.-100 (0.091 unit/mg protein) followed by G.Cot.-10 (0.102 unit/mg protein) under most salty condition (10 dS/m). As compare to control it was 0.087, 0.082, 0.097 and 0.096 fold higher in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively. Similarly at 45 DAS SOD activity was maximum in GISV-218 (0.138 unit/mg protein) followed by G. Cot.-16 (0.134 unit/mg protein) while it was lowest in G.Cot.-100 (0.104 unit/mg protein) followed by G.Cot.-10 (0.116 unit/mg protein). (Figure 5)

At both 15 and 45 DAS, highest percent increase Na⁺/K⁺ ratio in leaf was recorded in susceptible variety G.Cot.-10 (23%, 45%) and G.Cot-100 (33%, 37%) at the highest salt concentration as compared to their respective normal soil and least Na⁺/K⁺ ratio in G.Cot-16 (57%, 42%). (Figure 6)

The results of proline, phenol, POD, SOD, CAT and Na⁺/K⁺ ratio were in agreement with Ramani et al. (2018) [31] who screened eleven cotton genotypes that are grown up to squaring stage in different soil ratio of normal soil and saline soil. Proline content raised from 0.23 to 0.56 mg/g of tissue in normal soil condition and it was 0.75 to 1.35 mg/g of tissue in...
saline soil condition similarly highest total phenol content was found in GISV-218 and G. Cot-16 and Lowest total phenol content was found in G.Cot-10 and G.Cot-100. Further highest peroxidise activity was recorded in GISV-218 and G. Cot-16 while highest catalase activity was found in GISV-218 and Surat Dwarf in ratio of 1:2 (Normal soil: Saline soil). Highest Superoxide dismutase activity was found in GISV-218 and in ratio of 1:2 (Normal soil: Saline soil). Lowest Na/K ratio was found in GISV-218 and G. Cot-16 in root while lowest Na/K ratio was found in GISV-218 and G. Cot-16 in shoot.

At both the stages PPO enzyme activity was higher in tolerant genotype then in susceptible genotype. At 15 DAS it was highest in G.Cot.-16(4.16 unit/mg protein) followed by GISV-218 (3.63 unit/mg protein) while it was lowest in G.Cot.-10 (1.84 unit/mg protein) followed by G.Cot.-100 (2.50 unit/mg protein) under most saline condition (10 dS/m). As compare to control it was 39.40, 130.86, 246.27 and 195.39 percent increase in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively. Similar trend was observed at 45 DAS also. At 45 DAS PPO activity was highest in G.Cot.-16 (5.16 unit/mg protein) followed by GISV-218 (4.20 unit/mg protein) while it was lowest in G.Cot.-10 (2.54 unit/mg protein) followed by G.Cot.-100 (3.62 unit/mg protein) under higher saline condition. As compare to control it was 64.58, 180.16, 281.78 and 199.29 percent increase in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively (Figure 7). Earlier study by Dhimir & Kocacaliskan, (2001) [6] also showed significant correlation between NaCl concentration and PPO activity. Niknam, et al., (2006) [29] opined that PPO together with other enzyme effectively remove the ROS formed under salt stress.

PAL enzyme activity at seedling and squaring was higher in tolerant genotype then in susceptible genotype. At 15 DAS it was highest in G.Cot.-16(50.25 unit/mg protein) followed by GISV-218 (5.00 unit/mg protein) while it was lowest in G.Cot.-10 (2.48 unit/mg protein) followed by G.Cot.-100 (2.86 unit/mg protein) under most saline condition (10 dS/m). As compare to control it was 80.59, 112.91, 230.61 and 233.78 percent higher in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively. Similar trend of PAL activity was maximum in G.Cot.-16 (6.22 unit/mg protein) followed by GISV-218 (6.15 unit/mg protein) while it was lowest in G.Cot.-10 (2.70 unit/mg protein) followed by G.Cot.-100 (3.04 unit/mg protein) under high salinity condition (10 dS/m) at 45 DAS. As compare to control it was 64.50, 89.24, 248.50 and 260.54 percent increase in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively (Figure 8). Miladinova et al. (2012) also correlated PAL activity with the salt tolerant capacity. Gholizadeh and Kohnehrouz (2010) [11] found that PAL increased quickly when exposed to high salt concentrations (150 mM NaCl) at 24h salt stress in both maize inbreeds. Gao et. al. (2008) [11], observed that PAL activity in the cotyledons of Jatropha curcas was linearly and positively correlated with increasing NaCl concentrations, but the peak activity in the cotyledons was observed at NaCl concentration of 150 mM and the highest activity increased by 117.2% compared to the control. Further they concluded that increased PAL activity could be a response to the cellular damage provoked by higher NaCl concentrations.

**Fig 7**: Change in Enzyme activity of Polyphenol Oxidase (PPO) in cotton leaves during salt stress

**Fig 8**: Change in Enzyme activity of Phenylalanine Ammonia Lyase (PAL) in cotton leaves during different levels of salt stress
The C4H enzyme activity measured in leaves of cotton at different salinity levels showed a significant increasing trend with the increase of salt concentration at both 15 and 45 DAS (Figure 9). At both the stages C4H enzyme activity was observed higher in tolerant genotype then in susceptible genotype. At 15, 45 DAS it was highest in G.Cot.-16 (3.91, 5.09 unit/mg protein) followed by GISV-218 (2.83, 3.58 unit/mg protein) while it was lowest in G.Cot.-10 (0.98, 1.36 unit/mg protein) followed by G.Cot.-100 (1.66, 2.42 unit/mg protein) under most saline condition (10 dS/m). As compare to control it was 110.69, 227.61, 574.70, and 411.77 percent amplified in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively. Our results are in correlation with Mizutani et al. (1997) [25], who proved C4H expression was clearly higher in roots than in leaves in Arabidopsis plant.

![Fig 9: Change in Enzyme activity of Cinnamate-4-hydroxylase (C4H) in cotton leaves during different level of salt stress](image)

At both the stages C3H enzyme activity was observed higher in tolerant genotype then in susceptible genotype. At 15 DAS it was highest in G.Cot.-16 (3.53 unit/mg protein) followed by GISV-218 (2.26 unit/mg protein) while it was lowest in G.Cot.-10 (0.88 unit/mg protein) followed by G.Cot.-100 (1.57 unit/mg protein) under most saline condition (10 dS/m). As compare to control it was 83.33, 247.07, 612.03 and 358.13 percent amplified in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively. At 45 DAS C3H activity was maximum in G.Cot.-16 (4.50 unit/mg protein) followed by GISV-218 (2.84 unit/mg protein) while it was lowest in G.Cot.-10 (1.21 unit/mg protein) followed by G.Cot.-100 (2.27 unit/mg protein) under high salinity condition (10 dS/m). As compare to control C3H activity was 121.21, 328.30, 728.88 and 429.16 percent increase in G. Cot.-10, G.Cot.-100, G.Cot.-16 and GISV-218 respectively (Figure 10). The increase C3H activity changes the composition of lignin inturn alters the stiffness of cellwall and then, provides tolerance to salinity (Moura, et al., 2010) [26].

![Fig 10: Change in enzyme activity of P-Coumaric acid-3-hydroxylase (C3H) in cotton leaves during different levels of salt stress](image)

**Molecular Analysis**

Expression analysis of key genes of phenylpropanoid pathway was carried out at 15 DAS and 45 DAS after treatment with different concentrations of salt in both treated and non treated plants. The intact RNA isolated is converted to cDNA and is subjected RT-PCR analysis with PAL, C4H1 and C4H2 gene activity. 

**PAL** gene expression of different cotton genotypes is presented in Figure 11. It was observed that **PAL** gene expression increased with increasing salt concentration. At 15 DAS, maximum increase in PAL gene expression was observed in G.Cot.-16 (6.11 fold) followed by GISV-218 (5.74 fold); while, least increase was found in susceptible varieties i.e. G.Cot.-10 (2.93 fold) and G.Cot.-100 (2.45 fold) at highest salinity level (10 dS/m) as compared to normal soil (0.8 dS/m). Similar kind of trend was observed at 45 DAS also, maximum increase in PAL transcripts were observed in G.Cot.-16 (7.36 fold) followed by GISV-218 (6.77 fold), while, least increase was found in susceptible varieties i.e. G.Cot.-100 (3.14 fold) and G.Cot.-10 (2.85 fold) at highest
salinity level as compared to normal soil. Similar kind of increase in PAL activity was reported by Ibrahim, et al. (2019) [17, 18] who observed the effect of drought and salinity, alone or in combination, on secondary metabolism-related enzyme activities using two cotton.

Fig 11: Comparative real time PCR results in fold expression ($2^{ΔΔCT}$) of PAL gene in leaves of cotton during salt stress

genotypes Zhongmian 23 (salt tolerant) and Zhongmian 41 (salt sensitive). They found that PAL expression was increased in Zhongmian 23 by five fold, eight fold and six fold under drought, salinity and D+S respectively as compared to control. Similarly, Jeong, et al. (2012) observed that PAL transcript was significantly induced by 200 mM NaCl treatment was maximum after 24 h in Kenaf seedlings. Induction of PAL gene by NaCl was also observed by Guo and Wang (2009) [13] in the tomato.

Expression analysis of another important gene, $C4H$ was performed using $C4H$ ($C4H1$ & $C4H2$) genes specific primers. The gene $C4H1$ & $C4H2$ was found to be up regulated in salinity treated cotton plants as compared to non-treated plants. It was observed that $C4H1$ gene expression increased with increasing salt stress. At 15 DAS, maximum increase in $C4H1$ gene expression were observed in G.Cot.-16 (4.96 fold) followed by GISV-218 (4.47 fold) while, least increase was found in susceptible variety i.e. G.Cot.-100 (1.66 fold) and G.Cot.-10 (1.38 fold) at highest salinity level as compared to control (Figure 12). Similar kind of trend was observed at 45 DAS also, maximum increase in $C4H1$ gene expression were observed in G.Cot.-16 (6.92 fold) followed by GISV-218 (6.32 fold) while, least increase was found in susceptible variety i.e. G.Cot.-100 (2.19 fold) and G.Cot.-10 (1.38 fold) at highest salinity level as compared to control (Figure 13). It was observed that $C4H2$ gene expression increased with increasing salt stress. At 15 DAS, maximum increase in $C4H2$ gene expression were observed in G.Cot.-16 (4.47 fold) followed by GISV-218 (3.56 fold) while, least increase was found in susceptible variety i.e. G.Cot.-100 (1.20 fold) and G.Cot.-10 (1.01) at highest salinity level as compared to control. Similar kind of trend was observed at 45 DAS also, maximum increase

Fig 12: Comparative real time PCR results in fold expression ($2^{ΔΔCT}$) of $C4H1$ gene in leaves of cotton during salt stress compared to control. Similar kind of trend was observed at 45 DAS also, maximum increase in $C4H1$ gene expression were observed in G.Cot.-16 (5.13 fold) followed by GISV-218 (4.72 fold) while, least increase was found in susceptible variety i.e. G.Cot.-100 (1.82 fold) and G.Cot.-10 (1.64 fold) at highest salinity level as compared to control.
The C4H1 and C4H2 are differentially expressed isoforms of C4H gene. The existence of C4H isoforms was previously reported in maize and alfalfa and this differential expression of C4H isoforms might reflect the specific need for certain compounds at a certain time or compartment. C4H2 expressed constitutively and seems to play the role of a ‘housekeeping’ gene in the phenylpropanoid pathway. Transcripts of C4H1 detected in wound induced response while C4H2 transcripts detected without wound also. Similar trend increase in fold expression of C4H1 and C4H2 isoforms is observed in our study. The obtained results are in comparison with Kim et al. (2013) they observed that C4H induction was increased for up to 12 h after treatment, followed by a decreased at 24 h after treatment and a subsequent increased at 48 h after treatment at 150-200 mM NaCl /treatment in kenaf seedlings. The coordinated increase in C4H and PAL may participate in the reinforcement of cell walls by increased deposition of the lignin building units (Monolignols) and coumarates in the stressed plants.

Conclusion
The plants grown under saline condition have the ability to biosynthesize more biochemical metabolites in comparison to plants growing under normal conditions; this might be happening as a defence mechanism in plants. Looking to the tolerant genotypes, which performed better in stress environment have more actively produced phenols, proline, ROS enzymes and the key enzymes of phenylpropanoid pathway also revealed that the pathway is active in stressed plants.

Fig 13: Comparative real time PCR results in fold expression ($2^{-\Delta\Delta CT}$) of C4H1 gene in leaves of cotton during salt stress

The C4H1 and C4H2 are differentially expressed isoforms of C4H gene. The existence of C4H isoforms was previously reported in maize and alfalfa and this differential expression of C4H isoforms might reflect the specific need for certain compounds at a certain time or compartment. C4H2 expressed constitutively and seems to play the role of a ‘housekeeping’ gene in the phenylpropanoid pathway. Transcripts of C4H1 detected in wound induced response while C4H2 transcripts detected without wound also. Similar trend increase in fold expression of C4H1 and C4H2 isoforms is observed in our study. The obtained results are in comparison with Kim et al. (2013) they observed that C4H induction was increased for up to 12 h after treatment, followed by a decreased at 24 h after treatment and a subsequent increased at 48 h after treatment at 150-200 mM NaCl /treatment in kenaf seedlings. The coordinated increase in C4H and PAL may participate in the reinforcement of cell walls by increased deposition of the lignin building units (Monolignols) and coumarates in the stressed plants.

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The plants grown under saline condition have the ability to biosynthesize more biochemical metabolites in comparison to plants growing under normal conditions; this might be happening as a defence mechanism in plants. Looking to the tolerant genotypes, which performed better in stress environment have more actively produced phenols, proline, ROS enzymes and the key enzymes of phenylpropanoid pathway viz; PAL, C4H and C3H in cotton as compared to salt sensitive genotypes. Further, the molecular study of genes of phenylpropanoid pathway also revealed that the pathway was more active during stress condition from this it can be concluded that under saline stress the secondary metabolite pathway especially phenylpropanoid pathway is active in cotton.

References
1. AOAC. Official methods of analysis. Arlington: VA, 1990.
2. Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM. Allelopathy and exotic plant invasion: from molecules and genes in species interaction. Science. 2003; 301:1377-1380.
3. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water stress studies. Plant Soil. 1973; 39:205-207.
4. Beyer WF, Fridovich I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Analytical Biochemistry. 1987; 161(2):559-556.
5. Davidonis G, Richard O, Indberg B, William J, Heitholt J. The Influence of Cotton Seed Weight on Fibers per Seed and Fiber property Uniformity. Journal of New Seeds. 2005; 7:1-13.
6. Dhimir Y, Kocacaklisak L. Effect of NaCl and Proline on polyphenol oxidase activity in bean seedlings. Biologia Plantarum. 2001; 44(4):607-609.
7. Dudareva N, Pichersky E, Gershenzon J. Biochemistry of Plant volatiles. Plant Physiol. 2004; 135:1893-1902.
8. Fryxell PA. A revised taxonomic interpretation of Gossypium L. (Malvaceae). Rhedea. 1992; 2:108-165.
9. Gao S, Ouyang C, Wang S, Xu Y, Tang L, Chen F. et al. Effects of salt stress on growth, antioxidant enzyme and phenylalanine ammonia-lyase activities in *Introptra curcas* L. seedlings. Plant Soil Environ. 2008; 54(9):374-381.
10. Gao W, Xu FC, Guo DD, Zhao JR, Liu J, Guo YW et al. Calcium-dependent protein kinases in cotton: insights into early plant responses to salt stress. BMC plant biology. 2018; 18(1):15.
11. Gholizadeh A, Kohnehrouz BB. Activation of phenylalanine ammonia-lyase as a key component of the antioxidative system of salt-challenged maize leaves. Brazilian Journal of Plant Physiology. 2010; 22(4):217-223.
12. Greenway H, Munns R. Mechanisms of Salt Tolerance in Nonhalophytes. Annual Review of Plant Physiology. 1980; 31:149-190.
13. Guo J, Wang MH. Characterization of the phenylalanine ammonia-lyase gene (SIPAL5) from tomato (*Solanum lycopersicum* L.). Molecular biology reports. 2009; 36(6):1579.
14. Hangeman M, Erddman N. cyanobacterial nitrogen metabolism and environmental biotechnology. (A. K. Rai, Ed.) Environmental stresses, 1997, 156-221.
15. Huang J, Gu M, Lai Z, Fan B, Shi K, Zhou YH et al. Functional analysis of the arabdosis PAL gene family in plant growth development and responce to envirnomental stress. Plant physiology. 2010; 153(4):1526-1538.
16. Ibrahim W, Ahmed IM, Chen X, Cao F, Zhu S, et al. Genotypic differences in photosynthetic performance, antioxidant capacity, ultrastructure and nutrients in response to combined stress of salinity and Cd in cotton. Biometals. 2015; 28(6):1063-1078.
17. Ibrahim W, Qiu CW, Zhang C, Cao F, Shujin Z et al. Comparative physiological analysis in the tolerance to
salinity and drought individual and combination in two cotton genotypes with contrasting salt tolerance. Physiologia plantarum. 2019; 165(2):155-168.

18. Ibrahim W, Zhu YM, Chen Y, Qiu CW, Zhu S et al. Genotypic differences in leaf secondary metabolism, plant hormones and yield under alone and combined stress of drought and salinity in cotton genotypes. Physiologia plantarum. 2019; 165(2):343-355.

19. Kim DS, Hwang BK. An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signaling of the defence response to microbial pathogens. Journal of experimental botany. 2014; 65(9):2295-2236.

20. La Camera S, Gouzerh G, Dhondt S, Hoffman L, Fritting B, Legrand M et al. Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways. Immunol reviews. 2004; 198:267-284.

21. Lowry O, Nira J, Rosebrough A, Lewis F, Rose JR. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193(1):265-275.

22. MM, SD G, RP. (n.d.). Enhancement of peroxidase, polyphenol oxidase and superoxide dismutase activities by triadimefon in NaCl stressed Raphanus sativus L.

23. Mahatma MK, Bhatnagar R, Mittal GK, Mahatama L. Phenol metabolism in downy mildew resistant and susceptible genotypes of pearl millet. Arobives of Phytopathology and Plant Protection. 2011; 44(7):623-636.

24. Malick CP, Singh MB. Plant Enzymology and Histoenzymology. New Delhi: Kalyani Publications, 1980.

25. Mizutani M, Ohta D, Sato M. Isolation of a cDNA and a genomic clone encoding cinnaamate 4-hydroxylase from Arabidopsis and its expression manner in planta. Plant Physiology. 1997; 113(3):755-763.

26. Moura J, Bonine C, Viana J, Dornelas M, Mazzafera P et al. Abiotic and biotic stresses and changes in the lignin content and composition in plants. Journal of Integrative Plant Biology. 2010; 52(4):360-376.

27. Muhammad S, Shafeeq-ur-rahman, Allah D, Muhammad SI. Effect of salinity stress on cotton growth and role of Marker Assisted breeding and agronomic practices (Chemical, biological and physical) for salinity tolerance. Scholars Report. 2018; 4(1):1-13.

28. Naderi S, Seraji M, Fakheri B. Effects of Salinity Stress on Proline, Phenolic Compounds and Activity of Antioxidant Enzymes in Dracocephalum moldavica L. Elixir Agriculture. 2015; 82:32648-32651.

29. Niknam V, Razavi N, Ebrahimzadeh H, Sharifizadeh B. effect of NaCl on biomass, protein and proline contents and antioxidant enzymes in seedlings and calli of two trigenna species. Biol. Plant. 2006; 50:591-596.

30. Padhiar V, Sharma S. Accumulation of capscin in seed, pericarp and placenta of Capsicum annum L. fruit. journal of plant biochemistry and biotechnology. 2008; 17(1):23-27.

31. Ramani HR, Vekariya VK, Patel DH, Solanki BG. Screening of cotton genotypes against salinity stress based on its physiological and biochemical responses. Agriculture Update. 2018; 13(2):128-138.

32. Reddy MM, Gasber EO. Nicotiana. bot. Gaz. 1971; 132:158-166.

33. Thimmaiah SK. Standard methods of biochemical analysis. New Delhi: Kalyani Publications, 1999.

34. Vogt T. Phenylpropanoid biosynthesis. Molecular plant. 2010; 3(1):2-20.

35. Yaser HM, El-afry MM, Tantawy DS, Alshaal T, Abdelaal KA. Effect of some osmoregulators on photosynthesis, lipid peroxidation, antioxidative capacity, and productivity of barley (Hordeum vulgare L.) under water deficit stress. Envirnomental science and polution research, 2018.

36. Zhang H, Blumwald, Eduardo. Transgenic salt tolerant tomato plants accumulate salt in foliage but not in fruit. Nature biotechnology. 2001; 19:765-768.