

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.910.382>

Rubisco Degradation, Glutathione Reductase Induction, Proline and Valine Accumulation in Contrasting Wheats under Sodium Chloride (NaCl) Induced Oxidative Stress Conditions

Santosh Kumari^{1*} and Vipin Kumar Verma²

¹Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi, India

²Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India

*Corresponding author

ABSTRACT

Keywords

Drought, Glutathione reductase, Hydrogen peroxide, Proline, Ribulose biphosphate carboxylase/oxygenase, Sodium chloride, Valine, wheats

Article Info

Accepted:
26 September 2020
Available Online:
10 October 2020

Proline accumulation is a striking feature of drought sensitive wheat cultivar HD2428 under control and salt stress conditions. HD2428 combats reactive oxygen species (ROS) using proline accumulation capacity under normal conditions for growth and development. This accumulation was enhanced by oxidative stresses (H₂O₂ and NaCl treatments) in flag leaves of HD2428. Proline contents were lowered under combination of the oxidative treatments in both wheats. Proline contents were low in drought tolerant wheat cultivar C306. Drought tolerant wheat cultivar C306 utilized Ascorbate-glutathione reductase (GR) located at chloroplast to prevent oxidative damage under normal growth conditions. Oxidative stress under sodium chloride salt (NaCl) treatment induced mitochondrial and cytoplasmic GR in flag leaves of HD2428; chloroplast and cytoplasmic GR in C306 flag leaves to combat excess of ROS. Stimulation of Ribulose biphosphate carboxylase/oxygenase large subunit (Rubisco LSU) in drought tolerant wheat cultivar C306 in root and shoot indicated transcriptional changes under salt stress. Alterations in proline levels and GR isoforms were correlated and suggested changes in GR at translational levels in chloroplast, mitochondria and cytosol.

Introduction

RuBP carboxylase is the most abundant leaf protein (40% of total leaf proteins) and 20-30% of total leaf nitrogen. Chloroplasts are the major site of protein degradation during senescence and oxidative stresses. RuBP carboxylase resides in chloroplast stroma and catalyzes photosynthetic CO₂ fixation and photorespiratory carbon oxidation. The

enzyme consists of eight small subunits encoded by nuclear genes and eight large subunits encoded by a single chloroplast gene (Coen *et al.*, 1977) and synthesized on chloroplast ribosome (Blair and Ellis, 1973). Small subunit of RuBP carboxylase synthesized on cytoplasmic polyribosome (Roy *et al.*, 1976) accounts for up to 10% of the total proteins in plants. The presence of abundant Valine, Serine and Proline is the

predominant feature of the precursor to the small subunit of RuBP carboxylase (Schmidt *et al.*, 1979). The transit sequence of precursor is cleaved off by an endoprotease to enter the chloroplast envelope and to form holoenzyme.

Reactive oxygen species (ROS) are by products of photosynthesis, respiration and photorespiration. Excess ROS produced under abiotic stresses damage enzymatic proteins involved in these physiological processes and leads to leaf senescence. Plants remobilize nitrogen from old leaves to young leaves and reproductive organs by adopting senescence. Over production and accumulation of superoxide (SOD) and hydrogen peroxide (H_2O_2) leads to oxidative stress during senescence and environmental stresses (water, salt, high temperature and high radiation). ROS conversion to hydroxyl radical (OH^*) accounts for their main toxicity and damage to DNA, RNA, proteins, lipids and cell membranes.

Salt stress leads to osmotic effect in short term and ionic effect in long term. The osmotic pressure due to salt reduces water uptake, cell expansion and plant growth. Sodium (Na^+) accumulation in long term affects cellular metabolism, photosynthesis, stomatal opening, and leaf area and total biomass accumulation. Water loss leads to stomatal closure, carbon starvation and reduced biomass accumulation followed by early leaf senescence due to ion toxicity. Plants adopt senescence in response to stresses to recycle nutrient to the growing organs for the survival of next generation under various stresses. ROS play a role in the complex signalling network of plant responses under stresses. ROS levels are tightly regulated by induction and detoxification mechanism. The H_2O_2 produced in oxidative burst triggers programmed cell death. H_2O_2 levels could act

as a signal and activate transcription factor and induces antioxidant enzyme catalase that protect the plants from excess of H_2O_2 production.

Superoxide dismutases (MnSOD and CuZnSOD) are the metal containing enzymes that reduce the superoxide radicals. Glutathione (GSH), carotenoids and ascorbate are non enzymatic antioxidants that limit the ROS production in plant metabolism. GR maintains the GSH: GSSG ratios in chloroplast, mitochondria, peroxisomes and cytosol under various stresses (Mittler, 2002; Noctor *et al.*, 2012).

Exogenous application of proline, glycinebetaine, jasmonic acid, salicylic acid, ascorbic acid, glutathione and hydrogen peroxide has been shown to enhance salt tolerance. Salt stress and water stress induced accumulation of proline, glutamine and branched chain amino acids have been shown in plants and proline is the most comprehensively studied. Branched chain amino acids (leucine, isoleucine and valine) accumulate in response to nitrogen starvation and abiotic stresses. Rubisco degradation, proline and valine accumulation in contrasting wheats under oxidative stress conditions was undertaken in the present study to understand how plants sense oxidative stress. Hydrogen peroxide sprays treatment and NaCl salt treatment was used to create oxidative stress in wheat in the present study. The approach will help to characterize the key amino acids in stress signalling in drought sensitive and drought tolerant cultivars of wheat.

Materials and Methods

Drought sensitive wheat cultivar, HD2428 and drought tolerant wheat cultivar- C306 were grown at different dates (November 15, 2017 and January 15, 2018) to expose them to normal and oxidative stress environment

under late sown conditions. Plants were grown in green house in earthen pots (size 30x30 cm) filled with sandy loam soil and farmyard manure in 3:1 under natural environment. Each pot was fertilized corresponding to 120, 90 and 60 kg ha⁻¹ of N, P and K, respectively. Plants were kept free from diseases. Twenty pots were used for H₂O₂ (10 mM) spray treatment, NaCl (200 mM) soil application and H₂O₂ (10 mM) spray treatment after five days of NaCl treatment.

Flag leaves at ear emergence stage were sampled and ground in liquid N₂, homogenised in methanol, evaporated to dry powder and dissolved in methanol (HPLC grade) for GC-MS analysis [GCMS-QP2010 Plus].

Glutathione reductase (GR) Assay

Fresh flag leaves samples were ground in liquid nitrogen and homogenized in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and 4% (w/v) PVP-40. The homogenate was centrifuged at 10000 g for 20 min at 4°C. The supernatant was used for protein estimation (Bradford 1976) and antioxidant enzyme (GR) activity staining (Foyer et al.1994) using equal amount of protein.

Proline was extracted from fresh flag leaves in sulfosalicylic acid and estimation was done following Bates *et al.*, (1973).

Rubisco large subunit (LSU)

gDNA was extracted using the DNA sure plant mini kit - Nucleo-pore. DNA was used as a template for amplification of Rubisco (LSU) gene in a polymerase chain reaction (PCR) using Forward primer 5'- TGG ATTCAA AGC TGG TGT TA- 3' and

Reverse primer 5'-TAC TCG ATT AGC TAC GGC AC- 3' (NCBI accession number AM087200).

The PCR reaction was performed with 35 cycles and annealing temperature of 58°C. The extension and denaturing temperatures were set at 72°C for 1 min and 94°C for 30 second as standard protocols.

Results and Discussion

Proline accumulation was dramatically high in drought sensitive wheat cultivar HD2428 (Plate 1) when compared with drought tolerant cultivar of wheat C306 under various oxidative stress treatments (control, H₂O₂, NaCl and NaCl+H₂O₂). Proline contents were raised considerably in flag leaf of drought sensitive wheat cultivar HD2428 under H₂O₂ spray treatment and NaCl salt treatment than control. The values indicated higher intensity of oxidative stress in flag leaves of HD2428 under H₂O₂ treatment than under salt treatment. Drought tolerant wheat cultivar C306 displayed a reduction in proline contents in flag leaves under NaCl treatment in comparison to control. Proline contents decreased in flag leaves of both wheats under NaCl + H₂O₂ spray arrangement.

Striking level of Valine – Branched chain amino acid (BCCA) was detected under favourable growth conditions in drought sensitive wheat cultivar HD2428 (Fig. 2) than drought tolerant wheat cultivar C306 (Fig. 3).

Valine accumulation was evident under unfavourable growth conditions (oxidative stress due to high light and temperature) in drought sensitive cultivar HD2428 (Fig. 2A) and drought tolerant wheat cultivar C306 (Fig. 3A). Valine disappeared in flag leaves of both wheats (Fig. 2C and 3C) under prolonged salt stress (NaCl). H₂O₂ treatment raised the levels of valine in flag leaves of

C306 (Fig. 3B) in contrast to HD2428 (Fig. 2B) and decline in proline under favourable conditions for growth. Hydrogen peroxide sprays treatment (10 mM) and NaCl (200 mM) treatments at vegetative stage inhibited growth (height) of contrasting wheats. During senescence under normal plant life cycle proline and valine control the carbon and nitrogen level via succinate (Fig. 2) and gamma amino butyrate (GABA) metabolic pathway in drought sensitive wheat cultivar HD2428 than C306 drought tolerant wheat cultivar C306 (Fig. 3).

H₂O₂ raised valine levels from 9% in control to 15% in flag leaves of C306 (Fig. 3 and 3A) that could indicate the protein degradation and onset of senescence under excess of ROS. Our data showed the involvement of methyl jasmonate (MJ) in senescence in HD2428 (Fig. 2) under normal growth and development environment. The octadecanoid behave like local response regulator (Schaller, 2001) of plant growth.

Chloroplast GR isoform in C306 were suppressed under H₂O₂ treatment when compared with HD2428 under NaCl+H₂O₂ arrangement and H₂O₂ spray treatment (Plate3.). Oxidative stress due to H₂O₂ spray treatment reduced cytosolic GR1 in flag leaves of both cultivars under NaCl+H₂O₂ treatment. Oxidative stress enhanced by NaCl treatment could activated chloroplast GR isoforms in C306 and cytosolic GR1 in both cultivars under favourable growth conditions. However, the balance between GR1 (cytosol) and GR3 (mitochondria) could be anticipated in proline accumulation (Scandalios, 2002; Mittler *et al.*, 2004). Salt sensitive plants show an imbalance in antioxidant defences and cellular injury due to lipid peroxidation (Foyer and Noctor, 2000).

Our study suggests that chloroplast GR2 isoform catabolism participate in valine

accumulation in C306 under H₂O₂ treatment. The contrasting GR isoforms activation were parallel to proline contents under single treatment of NaCl and NaCl + H₂O₂ arrangement in flag leaves of both wheats. Enhanced activation of GR2 and GR4 in flag leaves of C306 could have reduced proline and valine levels by *de novo* synthesis of the enzyme utilizing GSH in chloroplast under NaCl stress. The ascorbate - glutathione cycle functions chiefly in chloroplasts to scavenge H₂O₂ produced through light reactions of photosynthesis (Noctor and Foyer, 1998).

Environmental and metabolic parameter affected the accumulation of LSU of Rubisco in roots and flag leaves under control and NaCl salt treatment in both wheats. Proline and valine accumulation in flag leaf correlated with LSU biosynthesis in roots and flag leaves of HD2428 in control plants indicated senescence associated protein degradation from older leaves and mobilization of amino acids to the growing organs (roots and ear emergence). Reduced glutathione (GSH) is the predominant form of glutathione that is mobilized via phloem and is required in biosynthetic reactions. Valine was not displayed in HD2428 flag leaves under NaCl treatment and proline contents were raised considerably in concurrence with enhanced activation of GR1 (cytosol) and GR3 (mitochondria). These results indicated reduced valine accumulation as a result of osmotic effect of proline in stabilization of proteins and their RNAs.

Higher number of Rubisco LSU in drought sensitive wheat cultivar HD2428 (Plate2.) indicated the higher copies of small subunit of Rubisco that is under nuclear control. Rubisco LSU of root declined in synchrony with reduced proline usage in biosynthesis of Rubisco SSU in the cytosol of HD2428 under salt stress. Data clearly indicated the *de novo* synthesis of proline in the cytosol and its

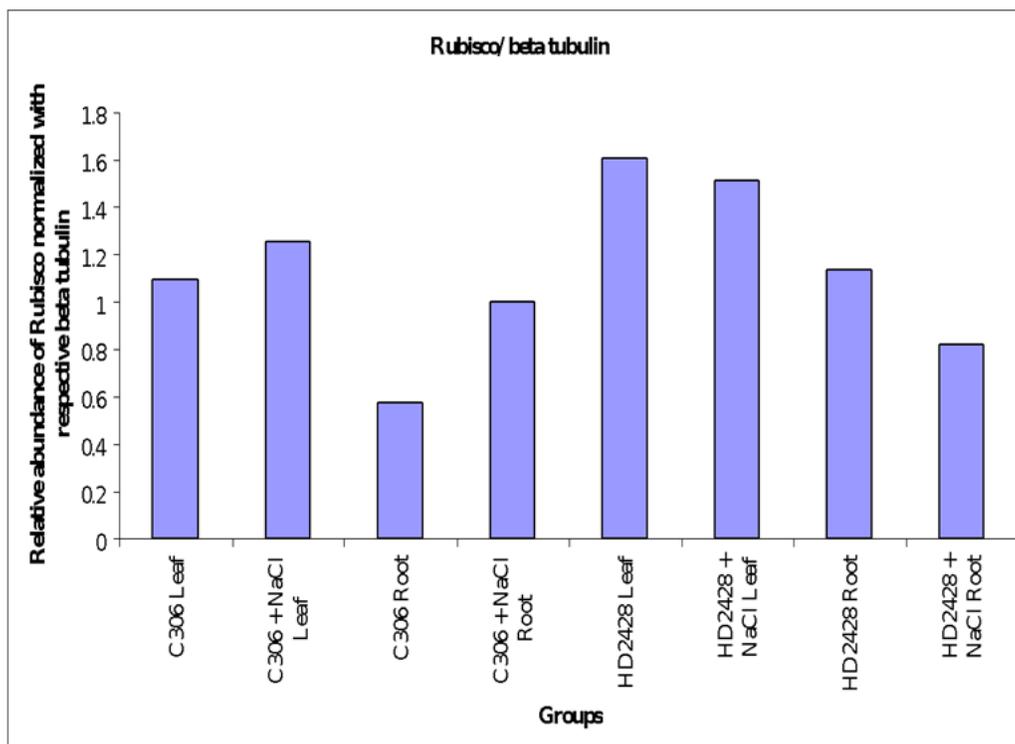
reduced incorporation in protein synthesis led to the amino acid accumulation. An increase in Rubisco LSU was evident in flag leaf and root of drought tolerant wheat cultivar C306 under salt stress (Fig. 1).

The two subunits of Rubisco undergo the coordinated and simultaneous synthesis and changes in their translatable messenger RNAs that can account for the change in the subunit biosynthesis. Therefore, increase in number of LSU in C306 under salt stress was related with biosynthetic activity and availability of GSH (Foyer and Noctor, 2000) for normal metabolism. Chloroplast and cytosolic GR isoforms in flag leaf of C306 (Plate 3) under NaCl treatment up regulated the redox homeostasis and activated synthesis of Rubisco LSU in leaves as well as roots. A decline of LSU in HD2428 roots further indicated the rise in oxidative stress in chloroplast and DNA damage under salt treatment. Proline is synthesized in cytosol

and catabolised in mitochondria. These changes were in concurrence with induction in cytosolic and mitochondrial GR isoforms in flag leaf of HD2428 under salt stress.

Inhibition of internodes elongation and changes in the biomass accumulation have been interpreted as an indicators of changes in endogenous gibberellin contents following cycocel treatment in wheat (Singh *et al.*, 1973). Proline accumulated in growth retardant treated wheat leaves under water stress. Growth promoter Gibberellin treatment reduced proline accumulation with increase in plant height and biomass accumulation in wheat. Drought sensitive semi dwarf wheat cultivar HD2428 exhibited the same trend under NaCl induced osmotic stress and H₂O₂ induced oxidative stress. Growth reduction of drought tolerant wheat cultivar C306 did not promoted proline accumulation under these treatments.

Fig.1 Rubisco relative expression in roots and leaves of drought sensitive cultivar HD2428 and drought tolerant wheat cultivar C306 under oxidative stress conditions (200 mM NaCl)



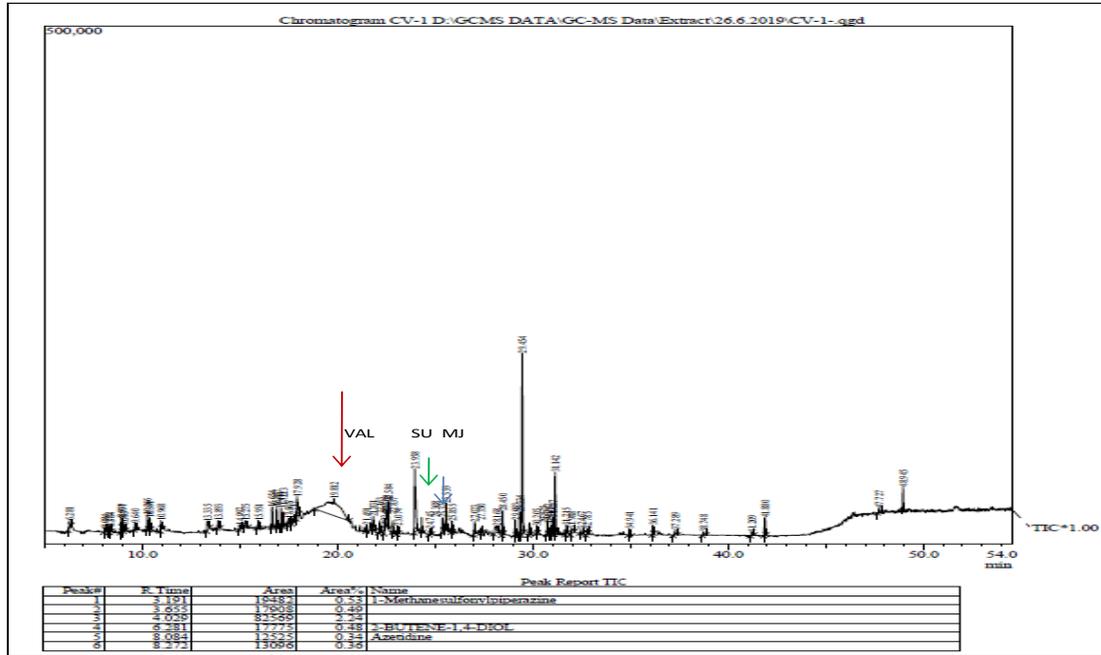


Figure 2. Chromatogram of drought sensitive wheat cultivar HD2428 ; flag leaf developed under favorable growth conditions (18 November 2018)

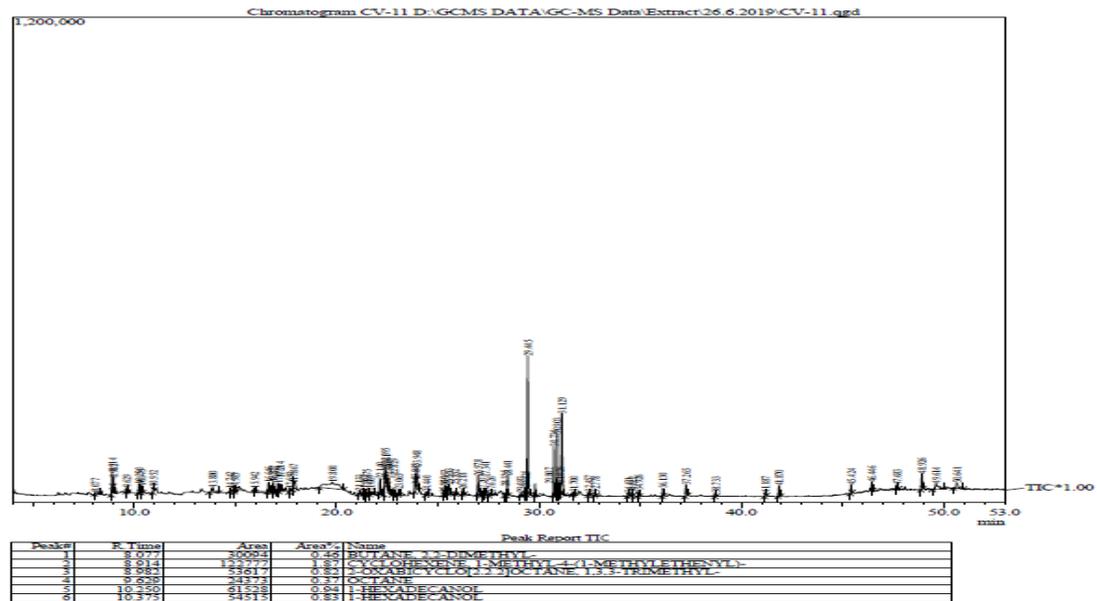
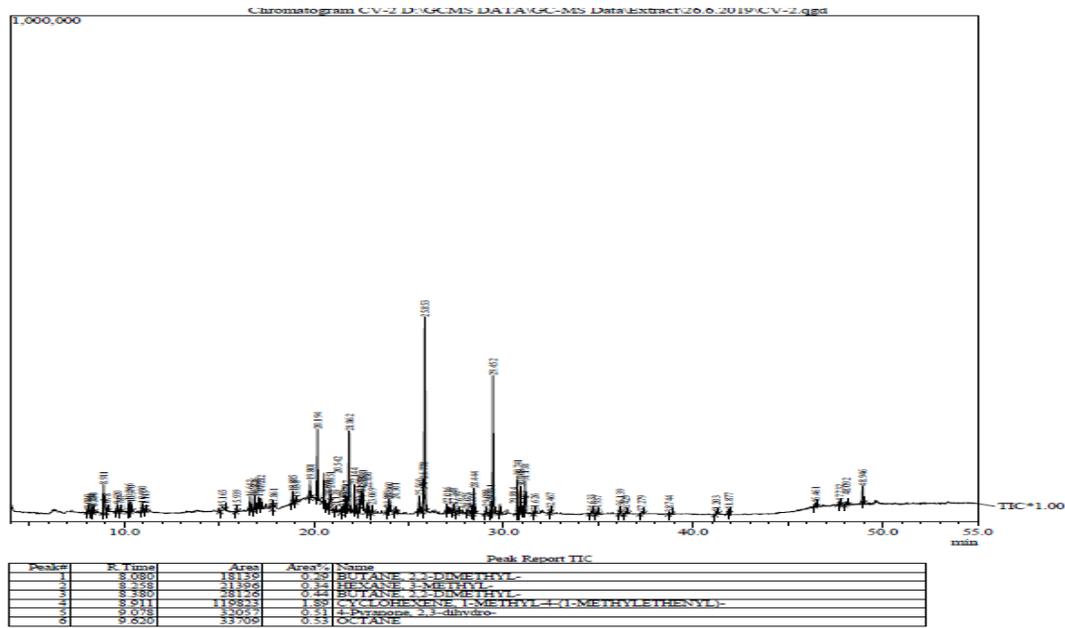


Figure 2A. Chromatogram of drought sensitive wheat cultivar HD2428 ; flag leaf developed under unfavorable growth conditions (18 January 2019)



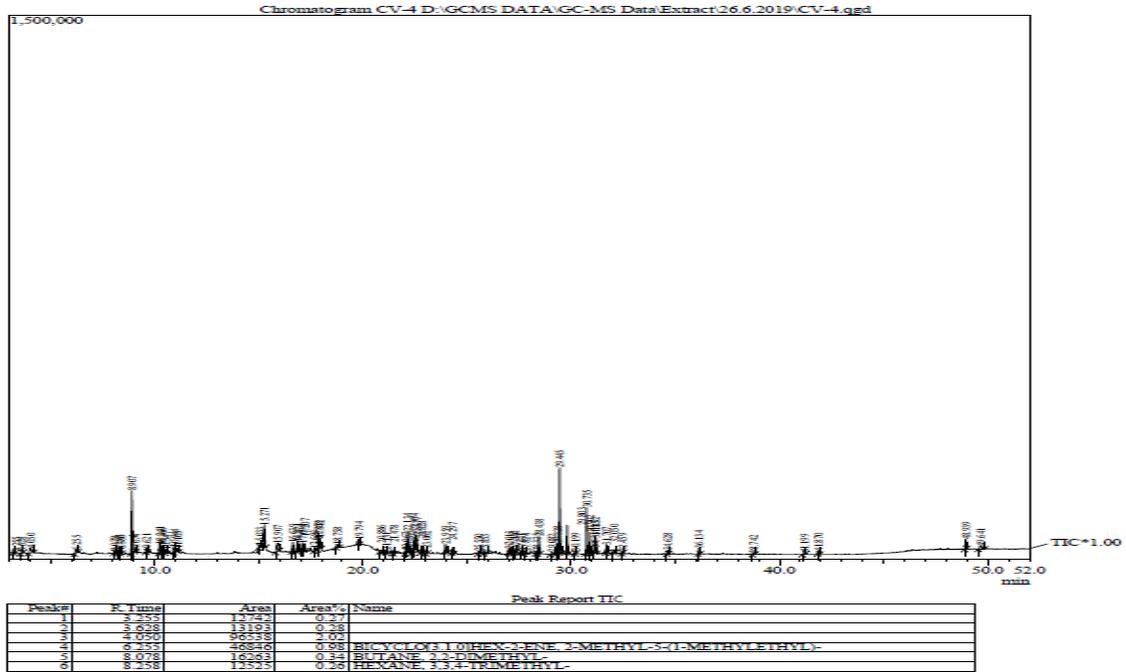
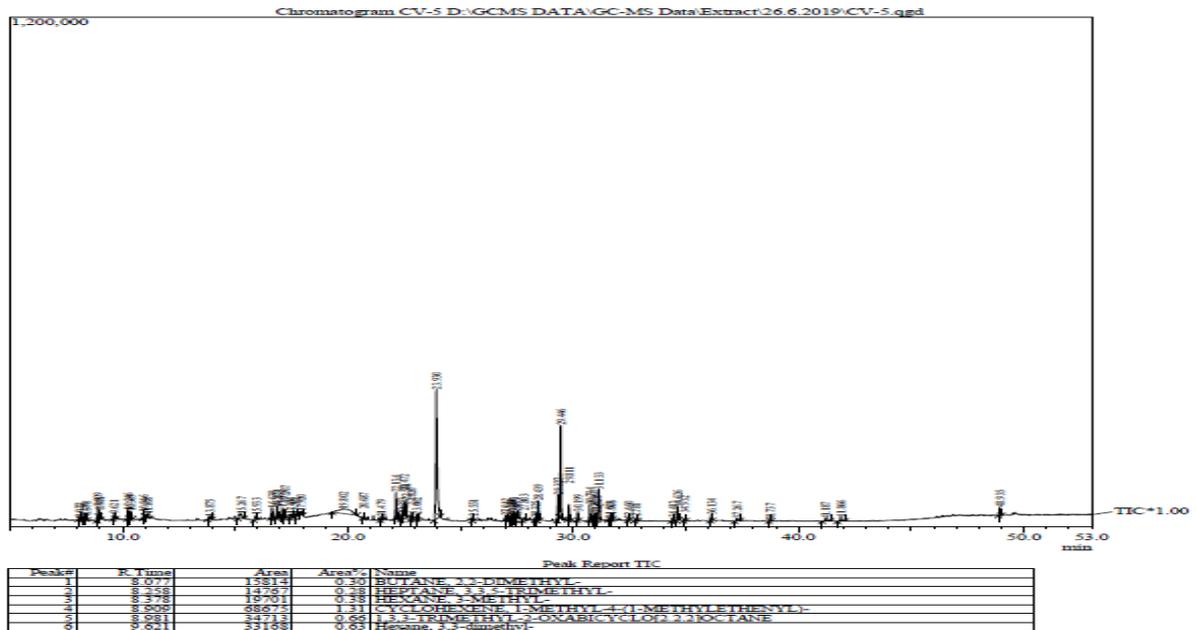


Figure 2D. Chromatogram of drought sensitive wheat cultivar HD2428 ; flag leaf developed under favorable growth conditions (18 November 2018) and NaCl treatment + H2O2 spray after 5 days at vegetative stage



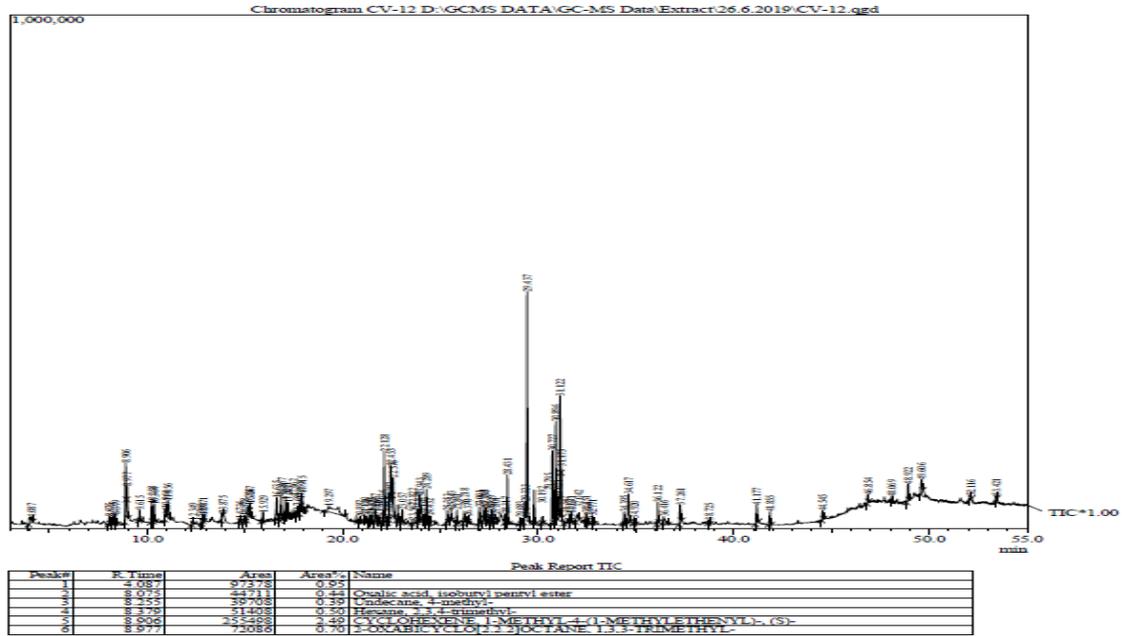


Figure 3A. Chromatogram of drought tolerant wheat cultivar C306; flag leaf developed under unfavorable growth conditions (18 January 2019)

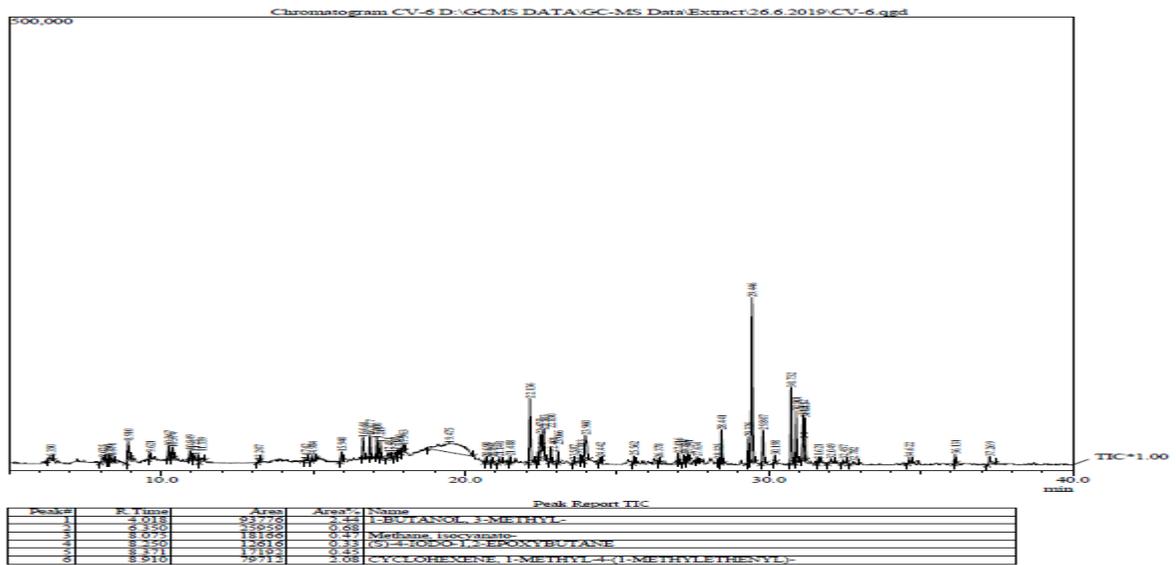


Figure 3B. Chromatogram of drought tolerant wheat cultivar C306; flag leaf developed under favorable growth conditions (18 November 2018) and H2O2 spray treatment at vegetative stage



| HD2428 | H ₂ O ₂ | NaCl | NaCl+ H ₂ O ₂ | C306 | H ₂ O ₂ | NaCl | NaCl+H ₂ O ₂ |
|----------|-------------------------------|--------|-------------------------------------|--------|-------------------------------|-------|------------------------------------|
| OD- 0.20 | 0.30 | 0.21 | 0.13 | 0.80 | 0.70 | 0.04 | 0.06 |
| 238 µg | 350 µg | 250 µg | 138 µg | 100 µg | 88 µg | 50 µg | 75 µg |

Plate1. Proline accumulation in flag leaves of drought sensitive wheat cultivar HD2428 and drought tolerant wheat cultivar C306 under oxidative stress conditions (200 mM NaCl)
 *Optical density (OD) Proline contents - µg per g fresh weight

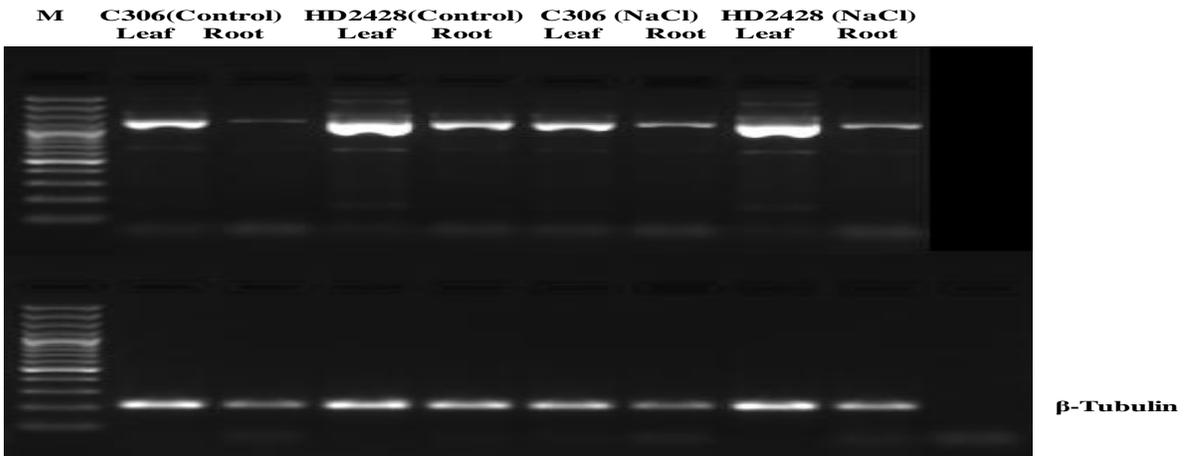


Plate2. Rubisco expression in root and leaves of drought sensitive wheat cultivar HD2428 and drought tolerant wheat cultivars C306 under oxidative stress conditions (200 mM NaCl)

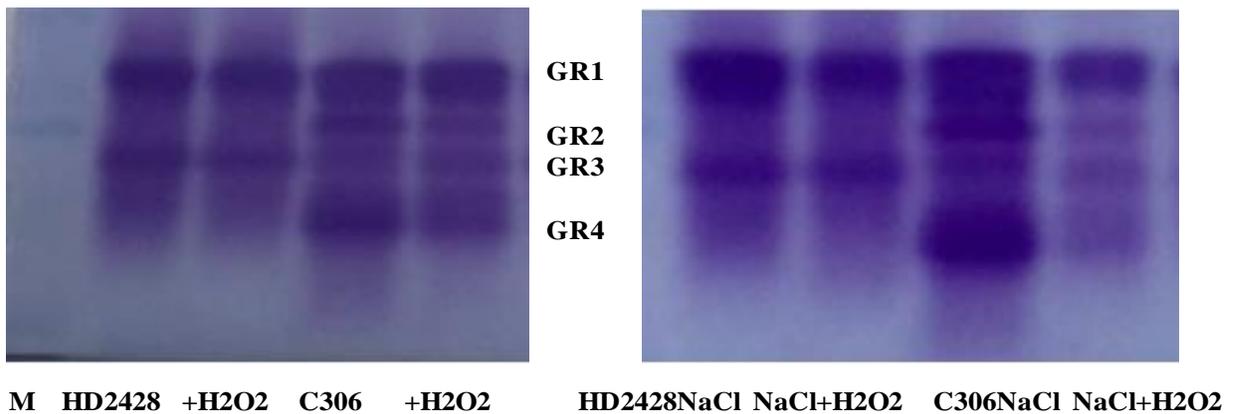


Plate3. Glutathione Reductase (GR) isozyme pattern in drought sensitive cultivar HD2428 and drought tolerant cultivar C306 under oxidative stress conditions (200 mM NaCl)

The carbon skeletons of amino acids are used in the intermediates of tricarboxylic acid ((TCA) cycle and ATP production under carbohydrate starvation and accelerated senescence (Däschner *et al.*, 2001, Araujo *et al.*, 2010). However, valine levels were correlated with large number of trichomes and secondary metabolism in HD2428 drought sensitive cultivar of wheat (Santosh Kumari and Verma 2020) under exogenous H₂O₂ treatment inducing oxidative stress.

H₂O₂ acts as a secondary messenger regulating growth and development as well as responses to various stresses. Hydroxyl radicals generated from H₂O₂ via Fenton reaction exerts toxic effects within all cellular compartments. Therefore, decreased GR isoforms in HD2428 flag leaf led to lipid peroxidation of chloroplast membrane, Rubisco protein degradation and proline and valine accumulation with the onset of senescence under normal growth conditions. Reduction of H₂O₂ by ascorbate-glutathione cycle helps in the adjustment of ATP/NADPH ratios and delay senescence of C306 flag leaves under drought/ osmotic effect of salt.

Our data indicated that a concerted ROS scavenging mechanism can be activated in chloroplast, cytoplasm and mitochondria. GR isoforms activation was positively related with ROS scavenging under salt stress in contrasting wheats. ROS play critical role in redox homeostasis, TCA cycle function and growth of wheat plant under NaCl induced oxidative stress in contrasting wheats. Nitrogen spared by reduced plant height is stored as proline in drought sensitive wheat cultivar. Drought tolerant wheat cultivar stored this spare nitrogen in the form of LSU of Rubisco in the chloroplast under osmotic stress that is mobilised under oxidative stress (H₂O₂) in the form of valine. Drought sensitive wheat cultivar had almost double number of Rubisco LSU under normal

conditions that declined under NaCl stress.

Acknowledgement

A GC-MS facility provided by AIIMS, Delhi is thankfully acknowledged.

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How to cite this article:

Santosh Kumari and Vipin Kumar Verma. 2020. Rubisco Degradation, Glutathione Reductase Induction, Proline and Valine Accumulation in Contrasting Wheats under Sodium Chloride (NaCl) Induced Oxidative Stress Conditions. *Int.J.Curr.Microbiol.App.Sci.* 9(10): 3192-3204. doi: <https://doi.org/10.20546/ijcmas.2020.910.382>