**The crosstalk of Melatonin and Hydrogen Sulfide Determines Photosynthetic Performance by Regulation of Carbohydrate Metabolism in Wheat under Heat Stress**

**Noushina Iqbal 1, Mehar Fatma 2, Harsha Gautam 2, Shahid Umar 1, Adriano Sofo 3,\*, Ilaria D’ippolito 3, Nafees A. Khan 2\***

1. Department of Botany, Jamia Hamdard, New Delhi-110062, India; noushina.iqbal@gmail.com (N.I.); s\_umar9@hotmail.com (S.U.)
2. Plant Physiology and Biochemistry Laboratory, Department of Botany, Aligarh Muslim University, Aligarh-202002, India; meharfatma30@gmail.com (M.F); harshagautam99@gmail.com (H.G.); naf9.amu@gmail.com (N.A.K.)
3. Department of European and Mediterranean Cultures: Architecture, Environment, Cultural Heritage (DiCEM), University of Basilicata, 75100 Matera, Italy; adriano.sofo@unibas.it (A.S); dippolito.ilaria@libero.it (I.D.)

\* Corresponding authors: naf9.amu@gmail.com (N.A.K); adriano.sofo@unibas.it (A.S.)

Materials and Methods Details

**Plant material and growth conditions**

Healthy seeds of wheat (*Triticum aestivum* L.) cultivar WH 542 were treated with 0.01% HgCl2 followed by washing with double distilled water to remove any adhered chemical after sterilization. Sterilized seeds were sown in pots filled with acid-washed purified sand. All pots were placed in an environmental growth chamber and day/night temperatures maintained at 25/18 °C, 12 h photoperiod (PAR 300 µmol m-2 s-1), and relative humidity of 65 ± 5%. Two plants per pot were maintained and were saturated every alternate day with 300 mL of full-strength Hoagland’s nutrient solution. The temperature stress treatment was given by subjecting the plants to 40 °C for 6 h daily for 15 d and were then allowed to recover at optimum temperature (25 °C) temperature and grown for the experimental period. The control plants were maintained throughout the experimental growth period at 25 °C.

**Leaf crude extracts for enzymatic assays**

Fresh leaves (200 mg) were homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) PVP in potassium phosphate buffer (100 mM, pH 7.0) using chilled mortar and pestle. The homogenate was centrifuged at 15,000×g for 20 min at 4 °C. The supernatant obtained after centrifugation was used for the activity assay of the different enzymes.

**Determination of H2S, H2O2 and TBARS content**

The content of H2S was measured in leaf by the formation of methylene blue from dimethyl-p-phenylenediamine in HCl. Leaves (0.7 g) were grounded in 2.5 mL of Tris-HCl (20 mM L–1, pH 6.8) containing 10 mM L–1 ethylene diamine tetraacetic acid (EDTA). The homogenate obtained was centrifuged at 4 °C and 12,000×g for 15 min. In the supernatant (0.75 mL), 0.2 mL of 1% (w/v) zinc acetate was added for trapping H2S. It was allowed to develop for 30 mins and then 0.1 ml of 20 mM L–1 dimethyl-p-phenylenediamine dissolved in 7.2 mol L–1 HCl and 0.1 mL of 30 mM L–1 ferric chloride in 1.2 mol L–1 HCl were added. The formation of methylene blue was determined spectrophotometrically at 670 nm. Different concentrations of NaHS were used as standard curve expressed as nmol g–1 fresh weight (FW).

For leaf H2O2 content, fresh leaves (500 mg) were ground in ice-cold 200 mM HClO4 (perchloric acid) to obtain homogenate which was centrifuged at 1,500×g for 10 min and then HClO4 of the supernatant was neutralized with the addition of 4 M KOH. Subsequently, another centrifugation was done at 500×g for 3 min to eliminate the insoluble KClO4.The reaction mixture contained 1.5 ml of eluate, 400 μl of 12.5 mM 3- dimethyl aminobenzoic acid (DMAB) in 0.375 M phosphate buffer (pH 6.5), 80 μl of 3-methyl-2- benzothiazoline hydrazone and 20 μl of peroxidase (0.25 units). The reaction was started by addition of peroxidase at 25 °C and the increase in absorbance was recorded at 590 nm.

For the content of TBARS (thiobarbituric acid reactive substances), fresh leaves (500 mg) were homogenized in 0.25% 2-thiobarbituric acid in 10% trichloroacetic acid and the mixture was heated at 95 °C for 30 min and then rapidly cooled on an ice bath. The mixture was centrifuged at 10,000×g for 10 min. To 1 mL aliquot of the supernatant, 4.0 mL of 20% trichloroacetic acid containing 5% thiobarbituric acid was added and final color intensity was recorded at 532 nm.

**Determination of Rubisco activity**

Rubisco activity was determined by monitoring NADH oxidation at 340 nm that occurs at 30 °C and after addition of enzyme extract to the reaction mixture, resulting in the conversion of 3- phosphoglycerate to glycerol-3-phosphate. For the extraction of the enzyme, 1.0 g of leaf tissue were subjected to ice-cold extraction buffer that comprised of 0.25 M Tris-HCl (pH 7.8), 0.0025 mM EDTA, 0.05 mM MgCl2, and 37.5 mg DTT and were homogenized in a chilled mortar and pestle. The homogenate was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant obtained after centrifugation was used to assay the enzyme. The reaction mixture contained 100 mM Tris-HCl (pH8.0), 10 mM MgCl2, 40 mM NaHCO3, 4.0 mM ATP, 0.2 mM NADH, 5.0 mM DTT, 0.2 mM EDTA, 1.0 U of glyceraldehydes-3-phosphodehydrogenase and 1.0 U of 3-phosphoglycerate-kinase and 0.2 mM of ribulose1,5-bisphosphate.

**Determination of Starch and total soluble sugars and sucrose content**

Dried leaf tissues were ground and filtered using a 1 mm sieve. The powdered material (0.1 g) was put into a 10 mL centrifuge tube and mixed with 5 mL of 80% ethanol. The mixture was incubatedin a water bath shaker at 80 ◦C for 30 min, and then centrifuged at 4000×g for 5 min.The pellets were extracted using 80% ethanol. Ethanol was removed through evaporation. Starch in the residuewas released in a boiling bath with 2 mL of distilled water for 15 mincooled to room temperature. Then, leaf starch was hydrolyzed with 9.2 mol L−1 HClO4 (2 mL) for 15 min. Distilled water (4 mL) wasadded into the samples, and samples were then centrifuged at 4000×g for 10 min. The residue was extracted one more timeusing 4.6 mol L−1 HClO4 (2 mL). The supernatants were retained, combined, and mixed with distilled water until 25 mL. The starch concentration was measured spectrophotometrically at A620 nm using an anthrone reagent and glucose was used as the standard.

For estimation of total soluble sugar, fully expanded top leaves were collected of each treatment. Leaf samples were oven-dried at 80 °C and ground to a fine powder. The dried sample (100 mg) was extracted using 10 mL of 80% ethanol and kept in a water bath at 80-85 °C for 30 min. The extract was centrifuged and the supernatant was transferred to a 100 mL volumetric flask, the extraction was repeated three times. Alcohol extract was evaporated on a water bath at 80-85°C. All the three supernatants were pooled in the flask following by addition of distilled water to 100 mL. Aliquot of the extract was used for determination of soluble sugars with anthrone reagent and the absorbance of reaction mixture was monitored at A630 nm using a spectrophotometer.

For sucrose content estimation each reaction contained 50 mM of UDP-glucose, 50 mM of extraction buffer, 10 mM of MgCl2 and 200 L of extract in a total volume of 550 L. The reaction was initiated by incubating the enzyme extract at 30 °C for 30 min. The reaction was stopped using 100 L 2 mol L−1 of NaOH and heating the solution for 10 min at 100 °C to destroy unreacted hexoses and hexose phosphates. The solution was then cooled and mixed with 1 mL of 0.1% (w/v) resorcin in 95% (v/v) ethanol and 3.5 mL of 30% (w/v) HCl before being incubated for 10 min at 80 °C. Sucrose content was calculated from a standard curve measured at A480 nm.

**Estimation of activity of sucrose synthase, sucrose phosphate synthase, acid invertase and UDP-glucose phosphorylas**e

Sucrose synthase and sucrose phosphate synthase activity assay were conducted at 37 °C in the direction of sucrose synthesis at pH 7.5. Two hundred microliters of desalted enzyme extract was added to 200 μl of assay solution. The sucrose synthase activity assay solution contained 50 mM Hepes (pH 7.5), 15 mM MgCl2, 25 mM Fru, and 25 mM UDP-Glc. The SPS assay solution contained 100 mM Hepes (pH 7.5), 20 mM Glc-6-P, 4 mM Fru-6-P, 3 mM UDP-Glc, 5 mM MgCl2, and 1 mM EDTA. For control, UDP-Glc was not added in the assay solutions. The reactions were kept for incubation of 30 min for SS and 60 min for SPS and then immediately stopped by boiling for 3 min. Enzyme reaction volume and freshly prepared reagents were proportionally increased for the spectrophotometric readings at 630 nm. The enzyme assay was measured in micromole per minute per gram of protein.

Acid invertase activity was assayed at 37 °C by adding 150 μL of desalted enzyme extract with equal volume of 1 M sodium acetate (pH 4.5) and 300 μL of 120 mM sucrose solution. The enzyme assay was expressed in micromoles per minute per gram of protein.

For determination of ADP-glucose phosphorylase, 0.5 g of dried leaves were homogenized in a prechilled pestle and mortar at 40 C with cold 2 mL of buffer on ice. The extraction buffer employed was having composition as 50 mM 3-N-morpholino propane sulphonic acid (MOPS) pH 7.4, 2 mM MgCl2 , 1 mM EDTA and 2 mM Dithiothritol (DTT) .The homogenate so obtained was centrifuged at 10,000 x g for 10 min in a refrigerated centrifuge at 40 °C. The supernatant was used as grain extract for enzyme analysis. The reaction was started by the addition of 200 µl of sodium pyrophosphate (2.5 µmole). The pyrophosphorolytic activity of ADP-GP was assayed spectrophotometrically by monitoring the increase in absorbance due to conversion of NADP to NADPH at 340 nm.