



Amelioration of heat stress during reproductive stage in rice by melatonin

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ABSTRACT

Melatonin is a low molecular weight hormone found in mammals and a natural bio-stimulating molecule in all living organisms from prokaryotes to eukaryotes. In plants melatonin plays an important role as a growth regulator and a stress buffering agent but its role under heat stress in rice reproductive stage remains undetermined. In the present study we have identified melatonin's role to alleviate heat stress mediated damages to photosynthesis system and chlorophyll damage in two contrasting genotypes for heat stress tolerance. High temperature stress was given at anthesis and the treatment of melatonin was applied as foliar spray. We observed that melatonin treatment significantly increased chlorophyll content under heat stress compared to mock treated plants. Further, our studies on photosynthetic traits gave an insight to melatonin mediated improvements on photosynthesis rate across all the treatments but more significantly in the thermo-sensitive genotype. Improved photosynthesis rate and chlorophyll content might be due to direct antioxidant scavenging and improved antioxidative system. All these findings show that melatonin has a potential role to develop crop varieties with higher stress tolerance capacity.

Key words: Heat stress, Melatonin, Photosynthesis, Reproductive stage, Rice

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine found in mammals, plants and all other living organisms. Melatonin was first discovered in the bovine pineal gland in 1958 by Aaron B. Lerner (Lerner *et al.* 1958). Melatonin helps in the regulation of circadian rhythms, sleep, mood and body temperature (Maronde and Stehle, 2007; Reiter *et al.* 2009 and Pandi-Perumal *et al.* 2008). In 1995 Dubbels and Hattori discovered melatonin in higher plants which is synthesised from tryptophan (Dubbels *et al.* 1995; Hattorie *et al.* 1995). In plant system melatonin acts as a growth regulator (Arnao and Hernandez 2015; Murch and Saxena 2002) and also have stress buffering capacity (Han *et al.* 2017).

In rice, flowering (anthesis and fertilization) and to a lesser extent booting (microsporogenesis) stages are the most susceptible stages affected by high temperature (Satake and Yoshida, 1978; Farrell *et al.* 2006). Heat stress affects structural organization of thylakoids, loss of grana stacking and swelling of grana under heat stress (Rodriguez *et al.* 2005, Ashraf and Hafeez, 2004). Photosystem II (PSII) activity is greatly reduced or even stops under high temperatures (Morales *et al.* 2003). The detrimental effects of heat on chlorophyll and the photosynthetic apparatus are associated with the production of injurious reactive oxygen species (Camejo *et al.* 2006; Guo *et al.* 2007).

Melatonin levels increased with increasing temperature increased when rice seedlings were exposed to various temperatures. Temperature-dependent melatonin synthesis was closely associated with an increase in both SNAT and ASMT activities (Byeon and Back 2014). At the reproductive stage of rice plant melatonin content was increased specially in panicle which indicates the importance of melatonin in this stage (Park *et al.* 2013). But the role of melatonin in heat tolerance mechanism at reproductive stage in rice is heretofore unexplored in the literature. In this study we have targeted photosynthesis related traits and chlorophyll degradation to understand the underlying physiological mechanism of melatonin mediated heat stress tolerance in rice.

MATERIALS AND METHODS

Planting materials and treatments: The planting of the material was done in the pot culture of Division of Plant Physiology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi during *kharif* season (crop duration: 16.06.2017-27.09.2017). The experiment was carried out with two contrasting genotype, i.e. thermo-tolerant NL 44 and thermo sensitive PB 1121 under complete randomised design (CRD) with three replications under each treatment combinations. For heat stress, plants were kept at 40.6°C (average) at anthesis stage and ambient plants at 32°C (average). Melatonin treatment of 200 µM was given as spray and applied in the morning one day before heat treatment. All the parameters were measured in flag leaf of rice plant.

Leaf Temperature Depression (LTD): Leaf temperature

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was measured by Infrared thermometer (AmiciKart® Digital Laser IR Infrared Thermometer-GM320) at 11 AM to 12 noon during the stress period. Five replications were taken from each biological replication for the study (Amani *et al.* 1996).

Chlorophyll content: Chlorophyll content was measured using the method described by Hiscox and Israelstam (1979) and calculated using the formula given by Arnon (1949). 50 mg fresh flag leaf samples were added to 10 mL DMSO and kept in dark for 4 h at 65°C. The absorbance was recorded at 663, 645 and 470 nm using DMSO as blank for calculating different pigment content.

Photosynthesis and related parameters: Net rate of photosynthesis was measured with an Infra-Red Gas Analyzer (IRGA), LI-6400 system (LI-COR, USA) using the method of Long *et al.* (1996). The flag leaf of the main shoot was used 3 days after stress from 1000 to 1200 hrs during the day time. Average air temperature during measurement was 35°C and ambient CO₂ was 410 ppm. The light intensity was fixed at 1000 μmol/m²/s. The net photosynthesis was calculated using ΔCO₂, flow rate and other factors such as the enclosed leaf area, the volume of enclosure and temperature. Photosynthesis rate was expressed as μmol CO₂/m²/s.

Quantum yield: Pulse Amplitude Modulated (PAM) chlorophyll fluorescence measurements were performed using a FluorPen FP100 growth chamber (Photo System Instruments, Czech Republic), on 30 min dark-adapted flag leaves. Light energy yields of the Photosystem II reaction centres were determined with a rational saturation pulse method as described by Bilger and Schreiber (1986).

Statistical analysis: The results are expressed as means with standard error (S.E.). The significance difference (at P < 0.05) between control and stressed samples were determined by Duncan's multiple range tests at a significance level of 0.05 for all biochemical and physiological parameters and was evaluated by analysis of variance (ANOVA). ANOVA and critical difference value were calculated by using SPSS 10.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel.

RESULTS AND DISCUSSION

Effect of melatonin on LTD: Leaf temperature and LTD was measured in two rice genotypes, viz. NL 44 (thermo-tolerant) and PB1121 (thermo-sensitive) under ambient and heat stress condition (Fig 1). In our study we observed that under heat stress canopy temperature increased in both the genotypes. PB 1121 genotype had a higher canopy temperature 37.3°C (+17.4%) than NL44 which was 35.8°C (+8.2%). Treatment of melatonin under heat stress showed a significant reduction in canopy temperature in NL 44 as well as PB 1121 genotype with 34.12°C (-4.6%) and 34.5°C (-7.3 %) respectively. When this data was expressed including ambient and high temperature, i.e. 33.6°C and 42.1°C respectively in terms of LTD we observed that under heat stress LTD was increased by 1062.5% in NL 44 and 166.4% in PB 1121. And melatonin treatment significantly increased LTD by 28.6% in NL44 and 63.6% in PB 1121.

Chaturvedi *et al.* (2017) observed that under heat

stress, tolerant variety had a higher rate of transpiration in comparison with susceptible genotype. From our data we observed that melatonin treatment helped to increase transpiration rate and thus reduced leaf temperature under stress. Finally we can conclude that melatonin increases transpiration rate under heat stress and helps to maintain

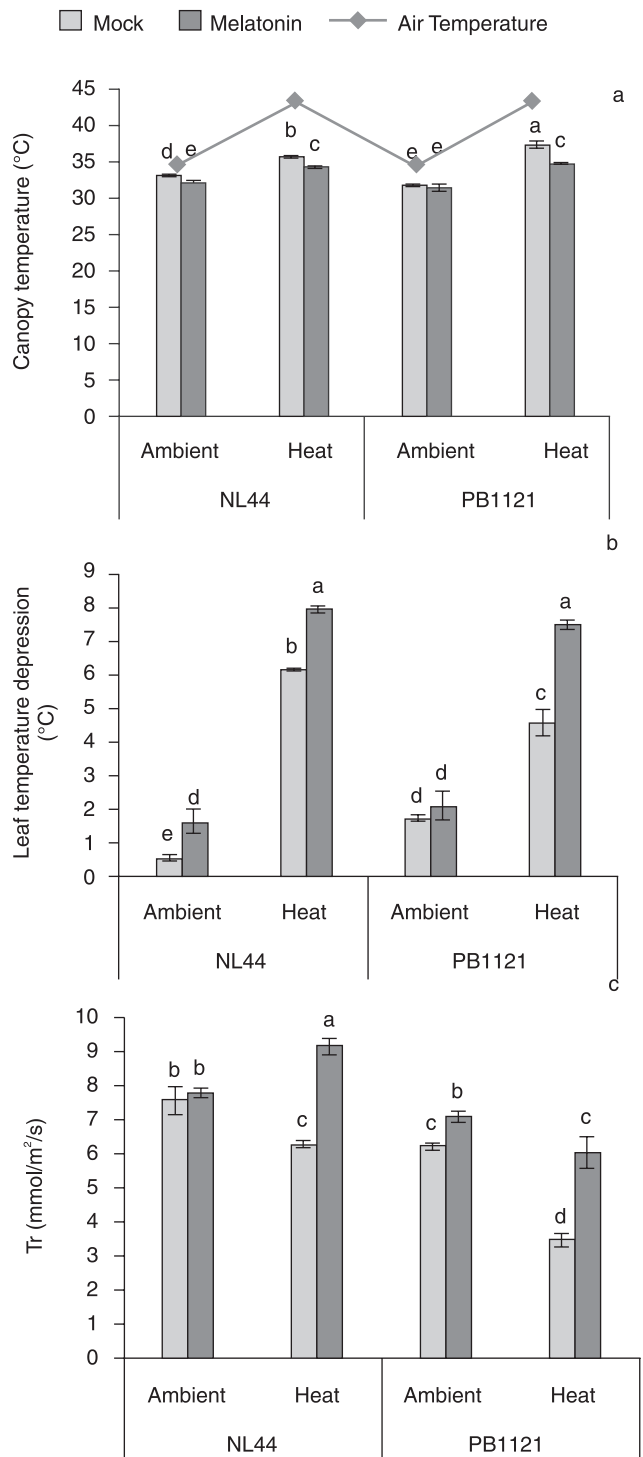


Fig 1 (a) Effect of melatonin treatment on leaf temperature, (b) leaf temperature depression and (c) leaf transpiration rate (Tr) in two contrasting genotypes viz. NL44 (thermo-tolerant) and PB1121 (thermo-sensitive) under heat stress.

comparatively lower leaf temperature for optimum biochemical activities.

Effect of melatonin on chlorophyll content: The photosynthetic function of leaf depends on light harvest pigments present in chloroplast. We found that under ambient condition NL 44 and PB 1121 had an average level of total chlorophyll content, i.e. 6.69 and 5.36 mg/g DW (Dry weight) but heat stress significantly reduced total chlorophyll content by 33.5% and 46.2% respectively (Fig 2). This reduction in total chlorophyll content under heat stress was greatly recovered by melatonin treatment. We observed increase of 31.1% in the thermo-tolerant and 55.3% increase in thermo-sensitive genotype. A similar trend was observed in chlorophyll *a* and chlorophyll *b* content under different treatments. Heat stress reduced chlorophyll *a* content by 32.3% in NL44 and 45.3% in PB1121 but melatonin treatment increased chlorophyll *a* content 5 mg/g DW (30.25%) in NL44 and 3.77 mg/g DW (50.11%) in PB1121. Heat stress reduced chlorophyll *b* content to 0.6 mg/g DW (40.6%) in NL44 and 0.76 mg/g DW (51.6%) in PB1121 whereas, melatonin treatment increased chlorophyll *b* content significantly about 0.82 mg/g DW (36.4%) in NL44 and 0.70 mg/g DW (90.4%) in PB1121. When we calculated chlorophyll *a/b* ratio we observed an increase of 6.3 (14.1 %) in NL44 and 11.9 (11.9%) in

PB1121 under heat stress but melatonin treatment reduced this ratio to 6.06 (4.2%) in NL44 which was not significant and a significant reduction of 5.5 (18.9%) in PB1121 under heat stress. Heat stress increases chlorophyllase activity and decreases the amount of photosynthetic pigments leading to reduced plant photosynthesis and increased respiratory activity (Sharkey and Zhang 2010). Our result on total chlorophyll, chlorophyll *a* and chlorophyll *b* shows a significant decrease under heat stress which might be due to increased oxidative damage but melatonin reduced chlorophyll degradation under stress. All together our observation signifies that melatonin reduced chlorophyll damage and sustains its activity under heat stress which may be due to improved antioxidant capacity which needs further research.

Effect of melatonin on stomatal conductance (G_s): Under ambient condition NL44 showed 0.27 mol/m²/s stomatal conductance and PB1121 had 0.23 mol/m²/s. Melatonin treated plants showed a higher rate of stomatal conductance across all the treatments (Fig 3). But under heat stress PB1121 reduced its stomatal conductance drastically to 0.14 mol/m²/s (35.2%) while NL44 had no significant change i.e. 0.24 mol/m²/s (11.1%). Whereas melatonin treated heat stressed plants improved their stomatal conductance to 0.33 mol/m²/s (37.3%) in NL44 and 0.30 mol/m²/s (103.4%)

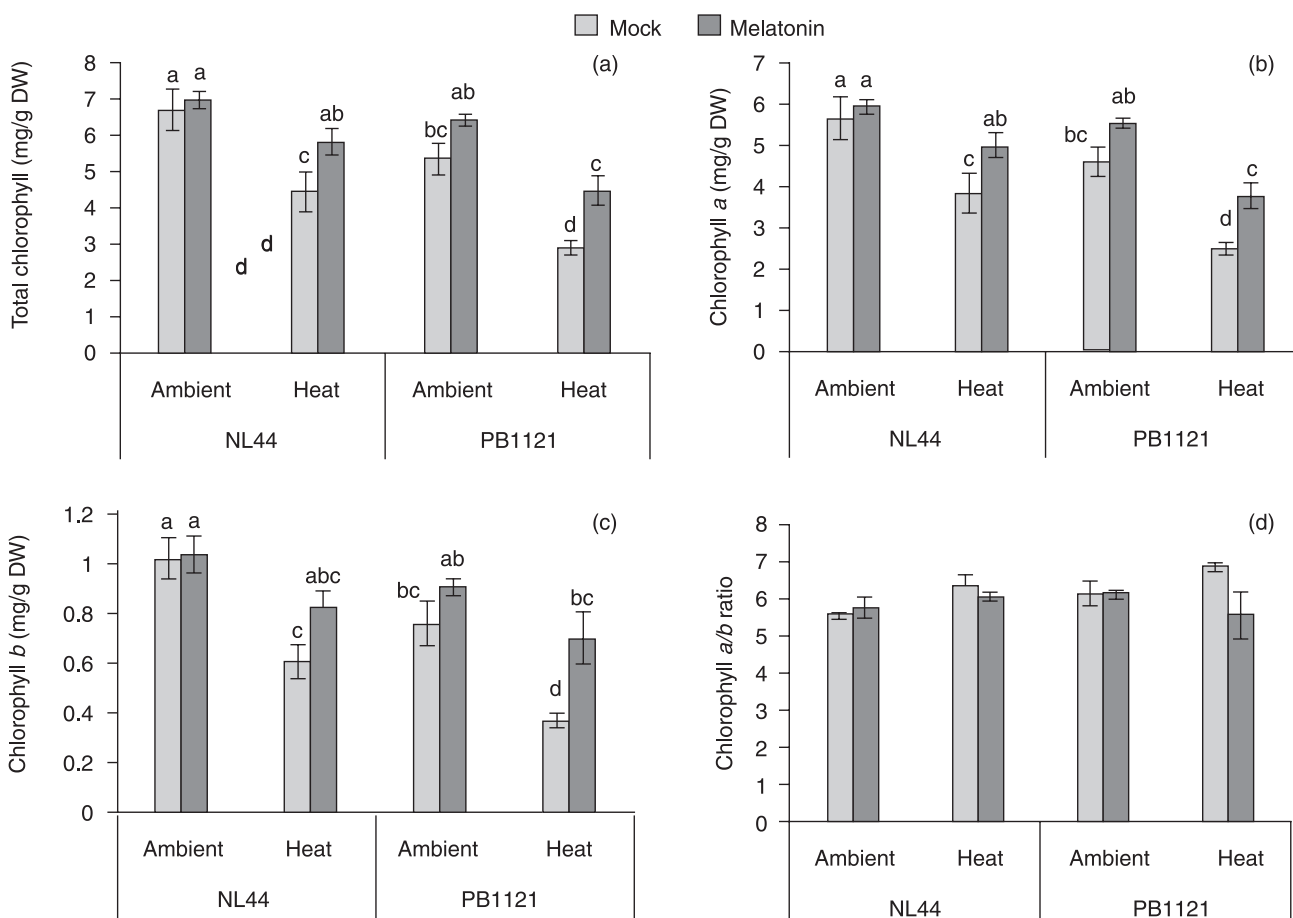


Fig 2 Effect of melatonin treatment on different photosynthetic pigments in two contrasting genotypes viz. NL44 (thermo-tolerant) and PB1121 (thermo-sensitive) under heat stress. (a) Effect of melatonin treatment on total chlorophyll content, (b) chlorophyll *a* content, (c) chlorophyll *b* content and (d) chlorophyll *a/b* ratio.

in PB1121. Previous studies have proved that heat stress reduce stomatal conductance severely in heat sensitive genotype compared to tolerant genotype (Chaturvedi *et al.* 2017). Our observations showed that melatonin treated plants had an increased stomatal conductance under all the treatments and this was more significant in PB1121. Previous work on tomato plant under stress had shown that melatonin increases stomatal conductance (Martinez *et al.* 2018). Higher rate of conductance with melatonin provides higher availability of CO₂ for photosynthesis. Our experiment also signifies melatonin's role in stomatal movement.

Effect of melatonin on internal CO₂ content (C_i): Internal CO₂ content in mock treated ambient plants were 280.6 μmol/mol and 391.2 μmol/mol in NL44 and PB1121 respectively (Fig 3). But heat stress had a positive effect on internal CO₂ concentration, i.e. increased internal CO₂ to 359.9 μmol/mol (28.2%) in NL44 and 391.2 μmol/mol (44.9%) in PB1121. Interestingly melatonin treatment helped to reduce internal CO₂ in both the genotypes under heat stress. In NL44 melatonin had reduced C_i to 301.6 μmol/mol (16.2%) and 274.5 μmol/mol (29.8%) in PB1121.

Under heat stress the efficiency of photosynthetic machinery decreases and respiration rate increases. As a result, internal CO₂ utilization through photosynthesis decreases whereas CO₂ production through respiration increases along with increased stomatal closure which leads to higher internal CO₂ concentration. In our experiment we observed higher C_i under stress which was due to reduced photosynthesis rate, reduced stomatal conductance and might be supported by increased respiration rate. Melatonin reverses all the negative effects and increases photosynthesis and reduced C_i level under heat stress which was highly significant in PB1121. Zhang *et al.* (2017) has observed reduced C_i with melatonin treatment under cold stress which was due to improved photosynthesis rate.

Effect of melatonin on transpiration rate (Tr): Heat stress had a significant effect on transpiration rate of both the genotypes. Under ambient condition NL44 and PB1121 had transpiration rate of 7.5 mmol/m²/s and 6.2 mmol/m²/s (Fig 3). We observed a decrease in transpiration rate in NL44 6.2 mmol/m²/s (16.9%) and more drastically in PB1121 with 3.4 mmol/m²/s (44.4%) under heat stress condition. But treatment of melatonin increased transpiration

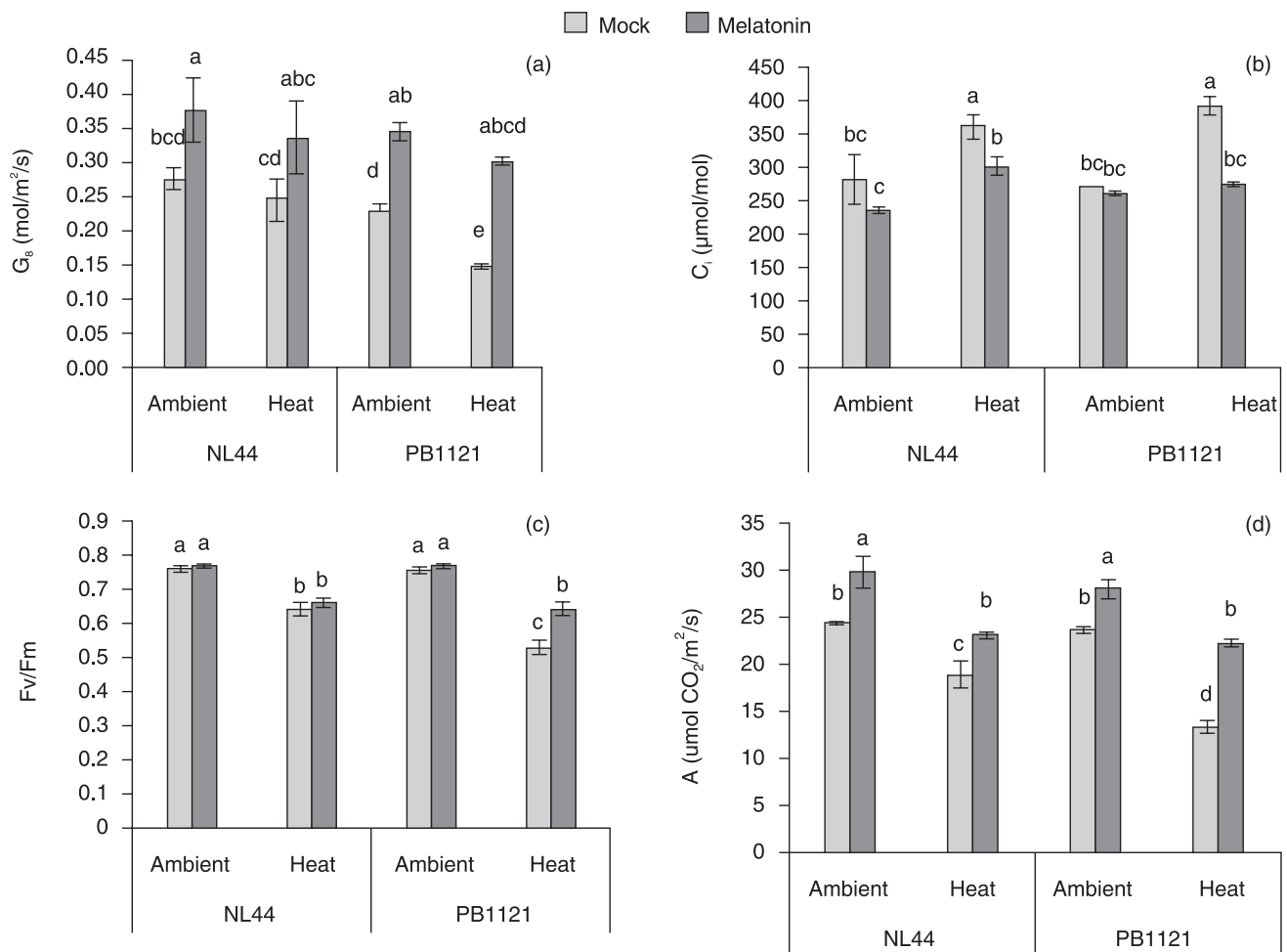


Fig. 3. Effect of melatonin treatment on different photosynthesis related parameters in two contrasting genotypes viz. NL44 (thermo-tolerant) and PB1121 (thermo-sensitive) under heat stress. (a) Effect of melatonin treatment on stomatal conductance (G_s), (b) internal CO₂ (C_i), (c) maximum quantum use efficiency of PSII (Fv/Fm) and (d) photosynthesis rate (A).

rate by 9.1 mmol/m²/s (45.48%) in NL44 and 6 mmol/m²/s (74.7%) in PB1121. Under heat stress transpiration rate decreases more in susceptible genotype due to reduced stomatal conductance and stomatal aperture (Chaturvedi *et al.* 2017). Due to stress there might be production of ABA which leads to reduced stomatal aperture and thus reduction in transpiration rate. But melatonin treatment increased transpiration rate against stress induced stomatal closure which is also supported by previous experiments (Zhang *et al.* 2017; Ahammed *et al.* 2018).

Effect of melatonin on maximum quantum use efficiency of PSII: Maximum quantum yield shows quantum use efficiency of photosystem and indirectly photosynthesis rate. Ambient rice plants showed quantum yield of 0.76 in both the genotypes (Fig 3). Whereas heat stress severely affected quantum use efficiency in PB1121 with 30.2% (0.53) reduction while 15.7% (0.64) reduction in NL44. In contrast melatonin significantly increased heat induced reduction in quantum yield by 21.3% (0.64) in PB1121 and negligible in NL44. Heat stress disintegrates thylakoid membrane structure by disturbing lipid bilayer (Pfeiffer and Krupinska 2005) and oxidizes proteins of oxygen-evolving complex (OEC) (Dias and Lidon, 2009; Rexroth *et al.* 2011), electron transport chain and dissociates the light-harvesting complex (Tang *et al.* 2007, Xue *et al.* 2011) which leads to decreased photosynthesis rate. Our result suggests that NL44 has acquired inbuilt mechanism to overcome heat stress induced damage but PB1121 lacks this, which was supplemented by melatonin treatment and stabilized PSII activity.

Effect of melatonin on photosynthesis rate (A): In ambient temperature NL44 had photosynthesis rate of 24.3 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ and 23.6 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ in PB1121 (Fig 3). Heat drastically reduced Photosynthesis rate with 18.8 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ (22.5%) in NL44 and 13.3 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ (43.6%) in PB1121. Whereas melatonin treatment showed significant increase in photosynthesis rate with 23 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ (22.3%) in NL44 and 22.1 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ (66%) in PB1121. Under heat stress condition high amount of reactive oxygen species (ROS) (Takahashi and Murata, 2006) is generated which oxidizes thylakoid membrane lipid and proteins of reaction center (Xu *et al.* 2006). All together heat induced damage to light harvest complex to electron transport chain caused reduction in photosynthesis. Under stress quantum use efficiency of photosystem reduces due to oxidative damage to PSII. But melatonin helped to reduce damage to PSII and increased maximum quantum yield thus increasing photosynthesis efficiency under heat stress which is also supported by previous findings with melatonin. (Zhang *et al.* 2017, Lazar *et al.* 2013).

Based on the experimental findings in our study it is concluded that melatonin has potential role to improve chlorophyll content and also helps to sustain photosynthetic efficiency under heat stress environment. The underlying mechanism behind higher chlorophyll and photosynthesis may be due to higher antioxidant scavenging by melatonin

and improved antioxidative mechanism. Further studies are required to identify or to develop melatonin enriched genotype for better abiotic stress tolerant crop.

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