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Exogenous nitric oxide alleviates high temperature induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by modulating the antioxidant defense and glyoxalase system

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Abstract

We investigated the protective role of exogenous nitric oxide (NO) in alleviating high temperature induced damages of wheat (*Triticum aestivum* L. cv. Pradip) seedlings. Heat treatment (38 °C) alone or in combination with 0.5 mM SNP (a NO donor) was applied with nutrient solution on 8-d-old hydroponically grown seedlings for a period of 24 h and 48 h. Heat stress significantly decreased the Chl content and increased the lipid peroxidation (MDA) and H_2O_2 levels in time depending manners. Ascorbate (AsA) content markedly decreased upon heat treatment but glutathione (GSH) and glutathione disulfide (GSSG) content increased. Heat treatment resulted in an increase of the activities of antioxidant enzymes - ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione *S*-transferase (GST). The activities of glyoxalase enzymes also increased upon heat stress. Exogenous NO supplementation in the seedlings had little influence on the nonenzymatic and enzymatic components compared to the control. However, supplementation of heat-treated seedlings with SNP significantly reduced the high temperature induced lipid peroxidation, H_2O_2 content and increased the content of Chl, AsA and GSH as well as the GSH/GSSG ratio. Heat treated seedlings which were supplemented with SNP also upregulated the activities of APX, MDHAR, DHAR, GR, GST, CAT and Gly I. This study concludes that an exogenous supply of NO protects wheat seedlings from high temperature induced oxidative stress by upregulating antioxidant defense and methylglyoxal (MG) detoxification system.

Keywords: Abiotic stress, Glutathione, Heat stress, Reactive oxygen species, Signaling molecules, Sodium nitroprusside.
Abbreviations: AO- ascorbate oxidase; APX- ascorbate peroxidase; AsA- ascorbic acid; CAT- catalase; CDNB- 1- chloro-2, 4-dinitrobenzene; Chl- chlorophyll; DHA- dehydroascorbate; DHAR- dehydroascorbate reductase; DTNB- 5,5′-dithio-bis (2-nitrobenzoic acid); EDTA- ethylenediaminetetraacetic acid; Gly I- glyoxalase I; Gly II- glyoxalase II; GR- glutathione reductase; GSH- reduced glutathione; GSSG- oxidized glutathione; GPX- glutathione peroxidase; GST- glutathione *S*-transferase; MDA- malondialdehyde; MDHA- monodehydroascorbate; MDHAR- monodehydroascorbate reductase; MG- methylglyoxal; NO- nitric oxide; NTB- 2-nitro-5-thiobenzoic acid; ROS- reactive oxygen species; SLG- *S*-D-lactoylglutathione; SNP - sodium nitroprusside; TBA- thiobarbituric acid; TCA- trichloroacetic acid.

Introduction

Plants often experience abiotic stress like salinity, drought, toxic metals, extreme temperatures, flooding, UV-radiations, ozone etc. Abiotic stress is responsible for the huge crop loss and reduced yield more than 50% of some major crops (Ahamad and Prasad, 2012; Hasanuzzaman et al., 2012a). One of the most important climatic factors that influence growth, development and yield of crops is temperature. High temperature response in plants becomes a major concern in the world because different global circulation models predict that greenhouse gases will gradually increase the world's average ambient temperature and lead to global warming (Meehl et al., 2007). High temperature or heat stress results from temperatures high enough to damage plant tissues, substantially influencing the growth and metabolism of plants (Balla et al., 2009). Although variable for different plant species, temperatures in the range of 35–45°C produce heat stress effects on tropical plants (Hall, 1992). The main symptoms of high temperature stress on plants may include scorching of leaves and twigs, sunburn on plant organs, leaf senescence and abscission, delay in seed germination and loss of vigor, imbalance in photosynthesis and respiration, reduction of shoot dry mass, relative growth rate and net assimilation rate, reduction in kernel growth and loss of kernel weight and density (Guilioni et al., 1997; Ismail and Hall, 1999; Egli et al., 2005; Wahid, 2007). In addition, heat stress, singly or in combination with drought, is a common constraint during anthesis and grain-filling stages in many cereal crops of temperate regions (Monjardino et al., 2005). High temperature in plants accelerates the generation and reactions of ROS including singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•), thereby induces oxidative stress (Mittler, 2002; Yin et al., 2008). Under high temperature, RuBisCO can lead to the production of H_2O_2 as a result of its oxygenase reactions

(Kim and Portis, 2004). The main effects of ROS include autocatalytic peroxidation of membrane lipids and pigments, modification of membrane permeability and functions (Xu et al., 2006). In addition to ROS, methylglyoxal (MG) is another cytotoxic compound which is highly accumulated in response to various extreme environmental stresses (Hasanuzzaman et al., 2011a, b; Hasanuzzaman and Fujita, 2011). Considering the drastic effects of extreme temperature, developing thermotolerance in crop plants is needed to sustain or increase the agricultural productivity for the increasing population of the world. The heat stress tolerance is an intricate phenomenon involving an array of physiological and biochemical processes at whole plant as well as molecular levels (Tiroli-Cepeda and Ramos, 2010). To counteract the toxicity of ROS a highly efficient antioxidative defense system, composed of both non-enzymatic and enzymatic constituents, is present in all plant cells. The enzymatic antioxidant defense mechanisms are represented by the enzymes, superoxide dismutase (SOD); 4 enzymes of the ascorbate-glutathione cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR); catalase (CAT); glutathione peroxidase (GPX); and glutathione *S*-transferase (GST). Non-enzymatic antioxidants include carotenoids, ascorbte (AsA), glutathione (GSH), tocopherol, flavanones, anthocyanins etc. (Noctor and Foyer, 1998; Gill and Tuteja, 2010). In line with ROS detoxification, the concentrations of MG must be kept under strict control because of its high cytotoxic and reactive properties. In plants, MG is detoxified mainly by the maintenance of GSH homeostasis via glyoxalase system (Yadav et al., 2005a, b) which comprises 2 enzymes, namely, glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I converts MG to *S*-D-lactoylglutathione (SLG), utilizing GSH, while Gly II converts SLG to D-lactic acid. During the latter reaction, GSH is regenerated. Different plant studies have demonstrated a clear role of the glyoxalase pathway in plants during exposure to stress. Numerous research findings support the notion that coordinated induction and regulation of the antioxidant and glyoxalase pathway enzymes are necessary to obtain substantial tolerance against oxidative stress (El-Shabrawi et al., 2010; Hasanuzzaman et al., 2011a, b; Hasanuzzaman et al., 2012b). Nitric oxide (NO) is one of the molecules which have received much attention during the last decade from plant researchers. It is considered as signaling molecule in many physiological processes during plant growth and development including seed germination, primary and lateral root growth, flowering, pollen tube growth regulation, fruit ripening, and senescence and so on. Nitric oxide also reported to provide protection of plants under different environmental stress conditions (Hasanuzzaman et al., 2010; Hasanuzzaman et al., 2011a; Corpas et al., 2011; Leterrier et al., 2012). Recently, NO oxide has been also considered as a new member of phytohormones (Leterrier et al., 2012). As NO is a highly reactive molecule and as a free radical it may scavenge other reactive intermediaries, it can counteracts oxidative stress by acting as an antioxidant, directly scavenging ROS (Laspina et al., 2005), or as a signaling molecule in a cascade of events leading to gene expression changes (Laspina et al., 2005; Hasanuzzaman et al., 2012c). In our previous report we also observed that NO could play important role in MG detoxification by upregulating the glyoxalase system enzymes (Hasanuzzaman et al. 2011a).

However, there are little or no study regarding the effect of exogenous NO on the antioxidant metabolism and MG detoxification system of plants under high temperature stress. Thus, the present study aimed to evaluate the effect of exogenous NO (SNP) on the activity of the antioxidant and glyoxalase pathway enzymes as well as on the levels of oxidative stress indicators, such as lipid peroxide, H_2O_2 levels and chlorophyll (Chl) content.

Results

Chlorophyll contents

As shown in Table 1, the Chl content in the wheat leaves decreased significantly under heat stress compared to the control. Compared to the respective control Chl *a* content was decreased by 15% and 31% at 24 h and 48 h, respectively. Similar to Chl *a*, Chl *b* content also decreased upon exposure to heat stress where the reduction was 25 and 22% at 24 and 48 h of heat stress, respectively compared to control. As a result, total Chl content of wheat leaves decreased by 18 and 28% at 24 and 48 h of heat treatment, respectively compared to respective control (Table 1). The ratio of Chl *a* and Chl *b*, however, remained unchanged due to heat or SNP treatment. Importantly, SNP supplementation in heat treatment significantly recovered the Chl depletion in heat stressed seedlings. In case of Chl *a*, both at 24 and 48h of heat stress, SNP treatment showed significant increase in the pigment content compared to heat alone. However, SNP supplementation did not show its effect in enhancing Chl *b* content in heat-stressed seedlings.

Lipid peroxidation and H_2O_2 level

Sharp increases in MDA content were observed in the seedlings exposed to 24 and 48 h of heat stress which were 76 and 144% higher than the respective controls (Fig. 1A). The SNP supplemented heat-stressed seedlings significantly lowered the levels of MDA compared to the seedlings exposed to heat alone. SNP supplemented heat stressed seedlings showed 27 and 31% reduction of MDA content compared with the heat stressed seedlings without SNP (Fig. 1A). A significant increase in the H_2O_2 level was observed in wheat leaves in response to heat stress and compared to control the levels was 61 and 141% higher at 24 and 48 h, respectively as compared to control (Fig. 1B). The SNP supplemented heat-stressed seedlings maintained significantly lower levels of H_2O_2 content (29 and 40% at 24 and 48 h of heat stress, respectively), as compared to the seedlings subjected to heat stress without SNP.

Non-enzymatic antioxidants

Marked decreases in AsA contents were observed (30 and 52% at 24 and 48 h, respectively) in response to heat stress, as compared to the control (Fig. 2A). Heat-stressed seedlings supplemented with SNP had significantly higher AsA content at any duration, as compared to seedlings subjected to heat stress without SNP. The AsA level of SNP supplemented stressed and non-stressed seedlings were similar to that of the untreated control. Drastic increases in GSH contents were observed in response to heat stress, as compared to the control (Fig. 2B). The seedlings exposed to 24 and 48 h of heat stress showed 90 and 153% increase in GSH content compared to their respective control (Fig. 2B).

Table 1. Chlorophyll contents of wheat leaves induced by high temperature, SNP and their combination. Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $P \leq 0.05$ applying DMRT.

Treatment		Chlorophyll content (mg g^{-1} fresh weight)			
		Chl *a*	Chl *b*	Total Chl	Chl *a*/*b* ratio
24 h	Control	0.494±0.019 a	0.244±0.017 a	0.738±0.021 ab	2.05±0.20 a
	SNP	0.521±0.009 a	0.237±0.017 a	0.758±0.015 a	2.23±0.18 a
	38 °C	0.418±0.030 b	0.184±0.013 b	0.602±0.031 d	2.29±0.27 a
	38 °C + SNP	0.475±0.020 a	0.203±0.015 ab	0.678±0.022 bc	2.37±0.22 a
48 h	Control	0.504±0.019 a	0.228±0.016 ab	0.732±0.022 ab	2.24±0.21 a
	SNP	0.535±0.007 a	0.239±0.017 a	0.774±0.019 a	2.27±0.17 a
	38 °C	0.346±0.023 c	0.177±0.013 b	0.523±0.030 e	1.97±0.16 a
	38 °C + SNP	0.413±0.006 b	0.203±0.015 ab	0.617±0.009 cd	2.06±0.17 a

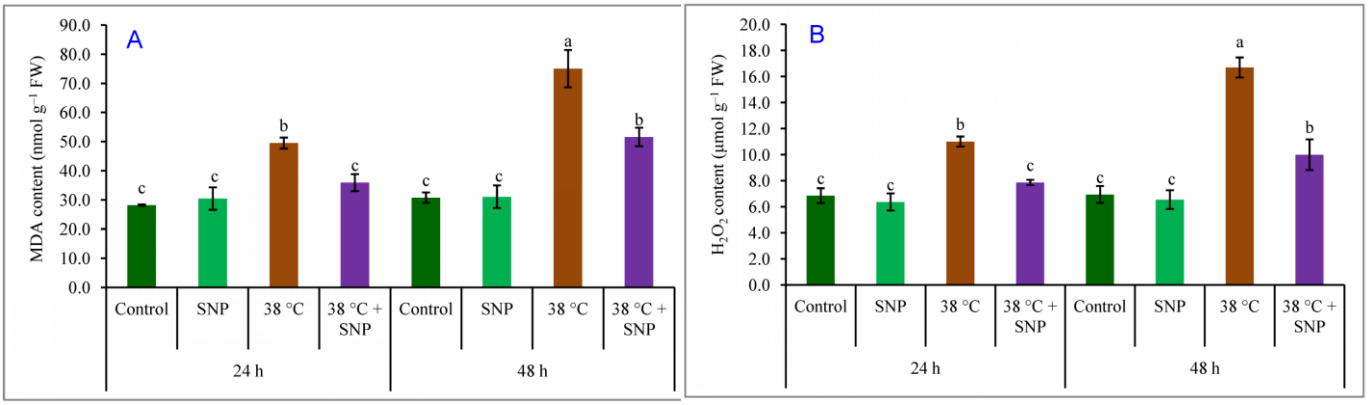

Fig 1. MDA (A) and H_2O_2 (B) levels in wheat leaves induced by high temperature, SNP and their combination. Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $P \leq 0.05$ applying DMRT.

Further increases in GSH content was observed in SNP supplemented seedlings grown under any extent of heat stress. GSSG contents in wheat leaves were increased by 167 and 210% at 24 and 48h of heat stress, respectively which was markedly higher than the control seedlings (Fig. 2C). On the other hand, the seedlings which were supplemented with SNP maintained the GSSG levels lower than the seedlings that were grown under high temperature without SNP supplementation (Fig. 2C). A significant decrease in the GSH/GSSG ratio was observed in response to heat stress which was 27 and 48% lower at 24 and 48h, respectively compared to control (Fig. 2D). However, for both of the cases, SNP supplementation maintained the ratio significantly higher than the heat stressed seedlings without SNP.

Activities of antioxidant enzymes

As shown in Fig. 3A, the APX activity of the wheat leaves increased under heat stress. The seedlings exposed to 24 and 48 h of heat treatment resulted in 59 and 44% increase of APX activity compared to control. In addition, further enhancement of APX activity was observed in SNP supplemented seedlings exposed to heat stress and compared to heat stress alone the activities of SNP supplemented seedlings were 29 and 23% higher (Fig. 3A). Compared to the respective controls, the activities of MDHAR was 31 and 48% lower in the seedlings exposed to 24 and 48 h of heat treatment, respectively (Fig. 3B). The DHAR activity was also decreased in the same way which was 26 and 40% lower at 24 and 48 h of heat treatment, respectively (Fig. 3C). SNP treatment alone had no effect on MDHAR activity but it slightly decreased the DHAR activity. More importantly, both the MDHAR and DHAR activities were enhanced in heat stressed seedlings which were supplemented with SNP and the activities were almost near or sometimes higher than the control (Fig. 3B, C). The activity of GR remained unchanged at 24 h of heat treatment, while it significantly increased (57%) upon 48 h of heat treatment (Fig. 3D). SNP supplementation only could enhance the GR activities at 24 h of heat treatment which was 37% higher than heat stress alone (without SNP). No remarkable effect of SNP was observed to enhance GR activity at 48 h of heat stress (Fig. 3D). Heat stress at any extent showed significant increase in GPX activities of wheat leaves. Upon exposure to heat for 24 and 48 h resulted in 48 and 65% increases in GPX activities compared to control (Fig. 4A). However, SNP supplementation in heat stressed seedlings could not enhance the activities further. Among all of the enzymes, GST activity of wheat leaves showed the most upregulation in response to heat stress. Upon 24 and 48 h of heat treatment the activity increased by 243 and 380%, respectively compared to control (Fig. 4B). More importantly, the activity in heat treated seedlings was further increased when supplemented with SNP. The CAT activity was slightly decreased under heat stress. At 24 and 48 h of heat treatment the activity decreased by 20 and 27%, respectively over control (Fig. 4C). At 24 h, SNP treatment alone also slightly decreased the CAT activity. In contrary, SNP supplementation combined with heat treatment at any extent significantly enhanced CAT activities compared to heat treatment alone (Fig. 4C).

Activities of glyoxalase enzymes

Slight increase in Gly I activity was observed in response to heat stress. As shown in Fig. 5A, heat treatment for 24 and 48 h resulted in 42 and 63% increase in Gly I activities

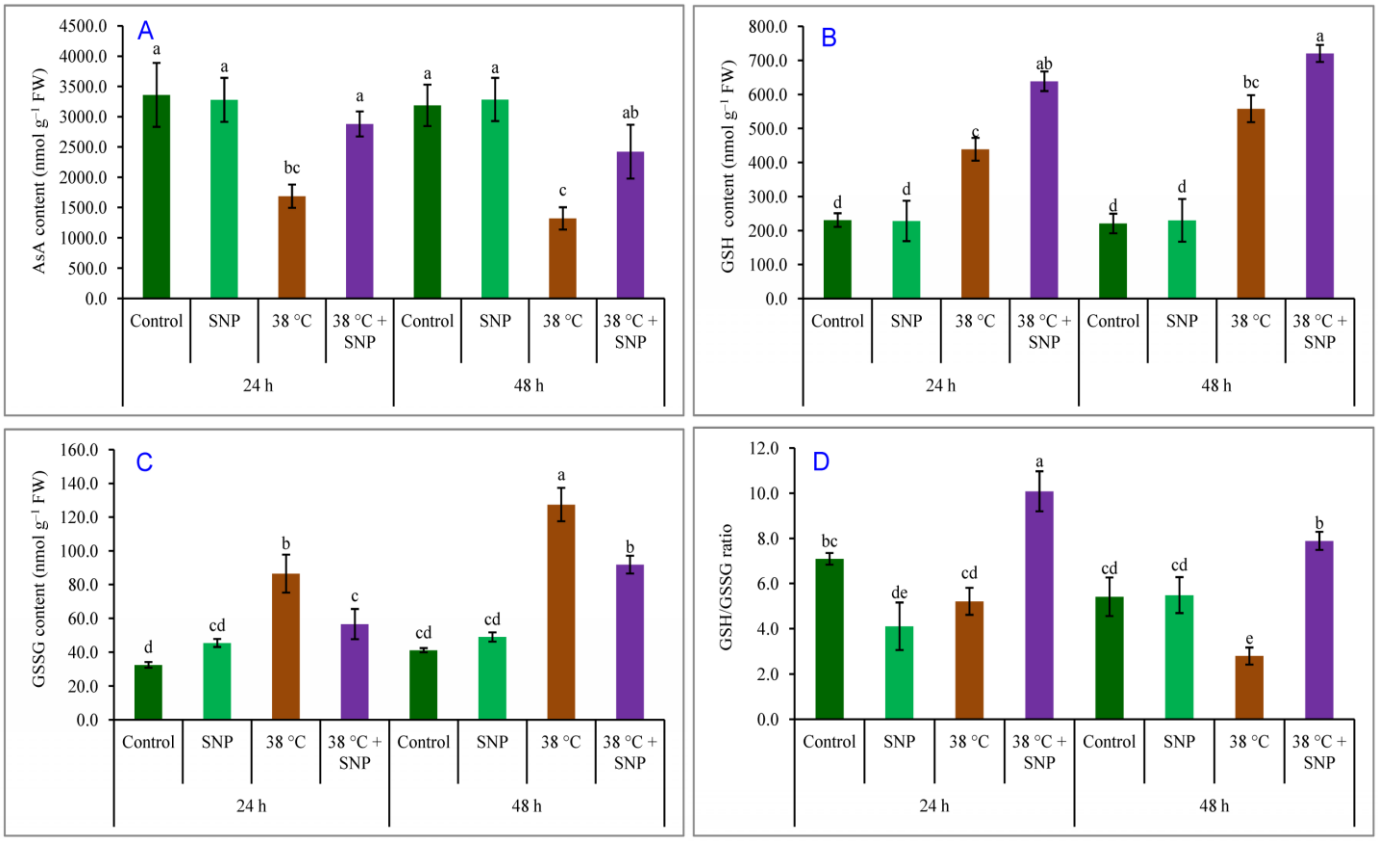

Fig 2. Contents of AsA (A), GSH (B), GSSG (C) and the GSH/GSSG ratio (D) in wheat leaves induced by high temperature, SNP and their combination. Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $P \leq 0.05$ applying DMRT.

compared to control. However, SNP supplementation in heat stressed seedlings caused more increase in the activities of Gly I compared to heat alone. The activities of Gly II was not changed upon 24 h of heat treatment but it significantly increased (96%) after 48 h of treatment (Fig. 5B). Compared to heat stress alone, SNP supplementation combined with heat treatment could not increase the Gly II activities further (Fig. 5B).

Discussion

Like other abiotic stress high temperature stress accelerates the generation and reactions of ROS including $^1O_2$, $O_2^{\bullet-}$, H_2O_2 and OH•, thereby inducing oxidative stress (Mittler, 2002; Yin et al., 2008). Under heat stress condition excess energy that has not been used for photosynthesis may produce large amounts of ROS, which may cause oxidative damage to chloroplasts and other cell structures (Asada, 1994; Singh and Singhal, 2001). High temperature stress also causes the reduction in Chl biosynthesis, disintegration of chloroplast membranes and disruption of the biochemical reactions of photosystems (Havaux, 1998). Our results indicated that heat stress significantly decreased Chl content in leaves which might be due to the impairment of Chl biosynthesis (Table 1). Similar impairment of Chl biosynthesis under high temperature was observed by several researchers (Efeoglu and Terzioglu, 2009; Aien et al., 2011; Reda and Mandoura, 2011). MDA is a well known index for determining the degree of oxidative stress. Its overall effects on plant cells are to decrease membrane fluidity; increase the leakiness of the membrane; and damage membrane proteins, enzymes, and ion channels (Garg and Manchanda, 2009). Hydrogen peroxide is a toxic compound which is injurious to the cell, resulting in lipid peroxidation and membrane injury (Pastori and Trippi, 1992). In our study, both the MDA and H_2O_2 levels significantly increased by heat treatment in time dependent manners (Fig. 1A, B). Addition of SNP in heat treated seedlings, on the other hand, reversed the effect and decreased the MDA and H_2O_2 content compared to heat treatment alone. This indicates that exogenous NO has a key role in ROS scavenging and reduction of oxidative stress in wheat seedlings subjected to heat stress. Similar reductions of MDA and H_2O_2 in SNP treated seedlings were observed in many plant studies (Song et al., 2006; Hasanuzzaman et al., 2011a; Kong et al. 2011). Non enzymatic antioxidants viz. AsA and GSH play key role in the antioxidative defense system (Foyer and Halliwell, 1976). AsA reacts with a range of ROS such as H_2O_2, $O_2^{\bullet-}$ and $^1O_2$, which is the basis of its antioxidant action. Additionally, GSH plays a key role in the antioxidative defense system by regenerating other potential water-soluble antioxidants via the AsA-GSH cycle. In addition, GSH plays a vital role in the antioxidant defense system as well as the glyoxalase system by acting as a substrate or cofactor for certain enzymes like GPX, GST and Gly I and thus participate in removal of ROS, MG and other endogenous toxic compounds (Noctor et al., 2012). Our results demonstrated that AsA content significantly decreased by heat treatment (Fig. 2A). This decreased was accompanied by the decreased activities of DHAR and MDHAR as well as increased APX activity (Fig. 3A–C).

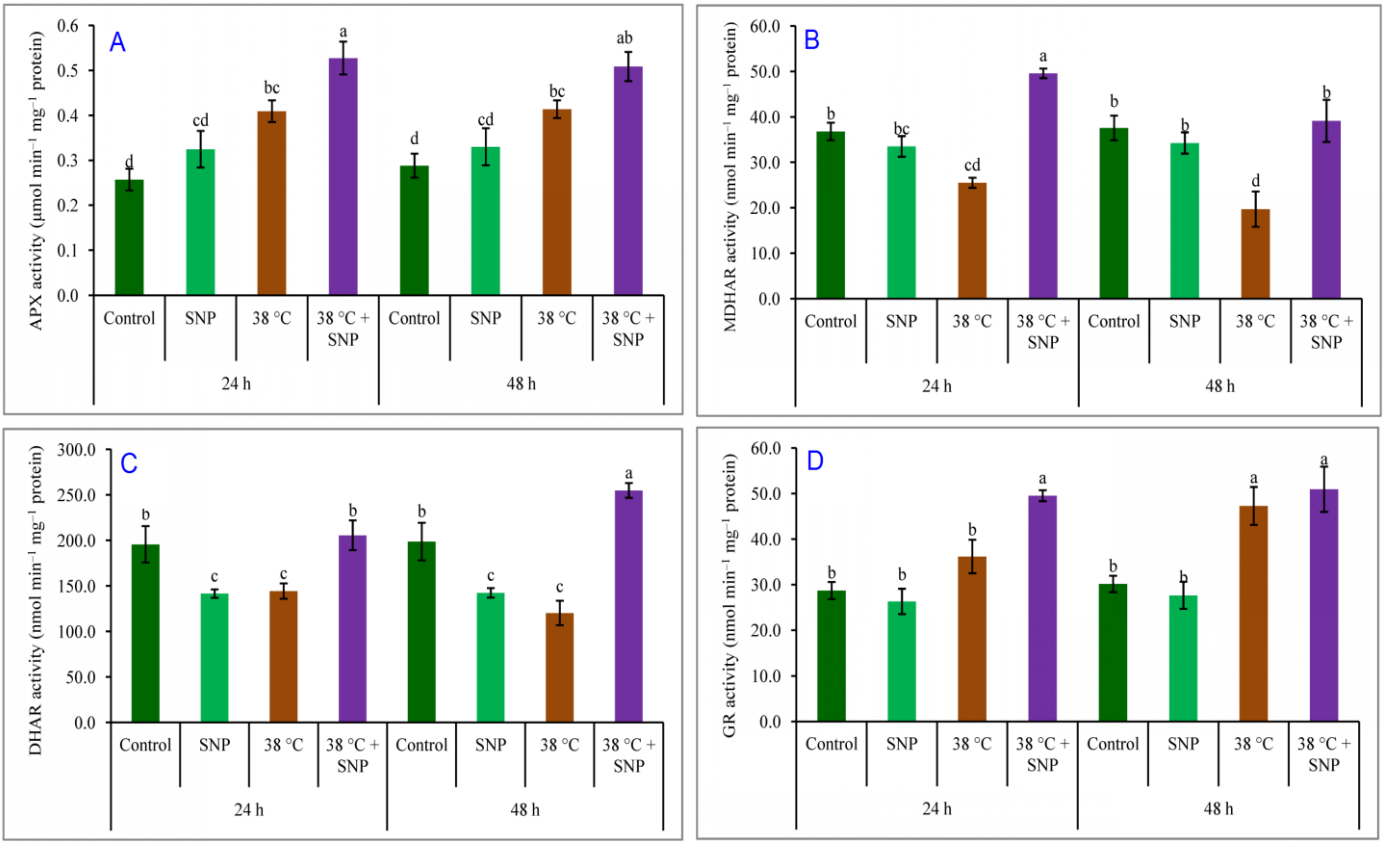

Fig 3. Activities of APX (A), MDHAR (B), DHAR (C) and GR (D) in wheat leaves induced by high temperature, SNP and their combination. Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $P \leq 0.05$ applying DMRT.

Glutathione in the leaves was highly accumulated during high temperature-induced oxidative stress (Fig. 2B). This result was supported by Kocsy et al. (2002) who found an increase of GSH content in plants subjected to heat stress which were due to a greater rate of GSH synthesis. In our study increased GSH content was also due to enhanced GR activity under heat stress (Fig. 3D). Both of the AsA and GSH content were significantly increased by SNP supplementation under heat stress condition which suggests that exogenous NO could participate in the synthesis or regeneration of AsA and GSH under heat stress. Similar results were observed in several plant studies (Tanou et al., 2009; Sheokand et al., 2010; Hasanuzzaman et al., 2011a). Increased GSH synthesis by NO treatment was reported by several groups (Innocenti et al., 2007; Xu et al., 2010). The formation of GSSG in heat-treated seedlings, on the other hand, might be due to the reaction of GSH with oxyradicals generated by oxidative stress or due to enhancement of GST activity that decomposes H_2O_2 and organic hydroperoxide. Increase of GSSG content under high temperature was reported by Rivero et al. (2004). Importantly, heat-treated plants supplemented with SNP decreased the GSSG content compared to heat treatment alone (Fig. 2C) which was due to the enhanced activity of GR (Fig. 3D). In our study, heat treatment resulted decrease in GSH/GSSG ratio which was significantly reversed by the supplementation of SNP. These results suggest that exogenous NO not only improves GSH biosynthesis or regeneration but also maintains their redox homeostasis. By nature, plants have developed enzymatic and non-enzymatic scavenging systems to quench ROS. When plants are subjected to stresses such as high temperatures, the scavenging system, in terms of antioxidant enzymes and metabolites, is not able to cope with the excess levels of ROS production, resulting in an imbalance in the production and quenching of ROS and consequently causes oxidative damage. The AsA-GSH cycle contains 4 enzymes: APX, MDHAR, DHAR and GR that are involved in the detoxification of H_2O_2. These enzymes also work in the regeneration of AsA and GSH. Our results demonstrated that APX and GR activities significantly increased with heat treatment in time dependent manners. The activities of MDHAR and DHAR, on the other hand, decreased markedly due to heat stress. These results were partially supported by Rivero et al. (2004) but opposite to some other previous results (Ma et al., 2008; Dai et al., 2012). The slight increase in APX and GR activities were not sufficient to protect the seedlings from ROS induced damages. As the MDHAR and DHAR are equally important in regulating the level of AsA and its redox state under oxidative stress (Eltayeb et al., 2006; 2007), these decreases in the activities were reflected by decreased AsA content. However, SNP supplemented seedling exposed to heat treatment significantly increased the activities of these enzymes except for GR activity at 48 h of heat treatment (Fig. 3A-D). The upregulation of AsA-GSH cycle enzymes by NO treatment was observed by Hasanuzzaman et al. (2011a). GPX is also a principal cellular enzyme capable of repairing membrane lipid peroxidation and is an important protectant against oxidative membrane damage (Kühn and Borchert 2002). Our results indicated a slight increase in GPX activity in response to heat stress which indicates its week participation in ROS scavenging. In addition, SNP supplementation also could not enhance the activity (Fig. 4A). The plant GSTs are a large and diverse group of enzymes that catalyze the conjugation of

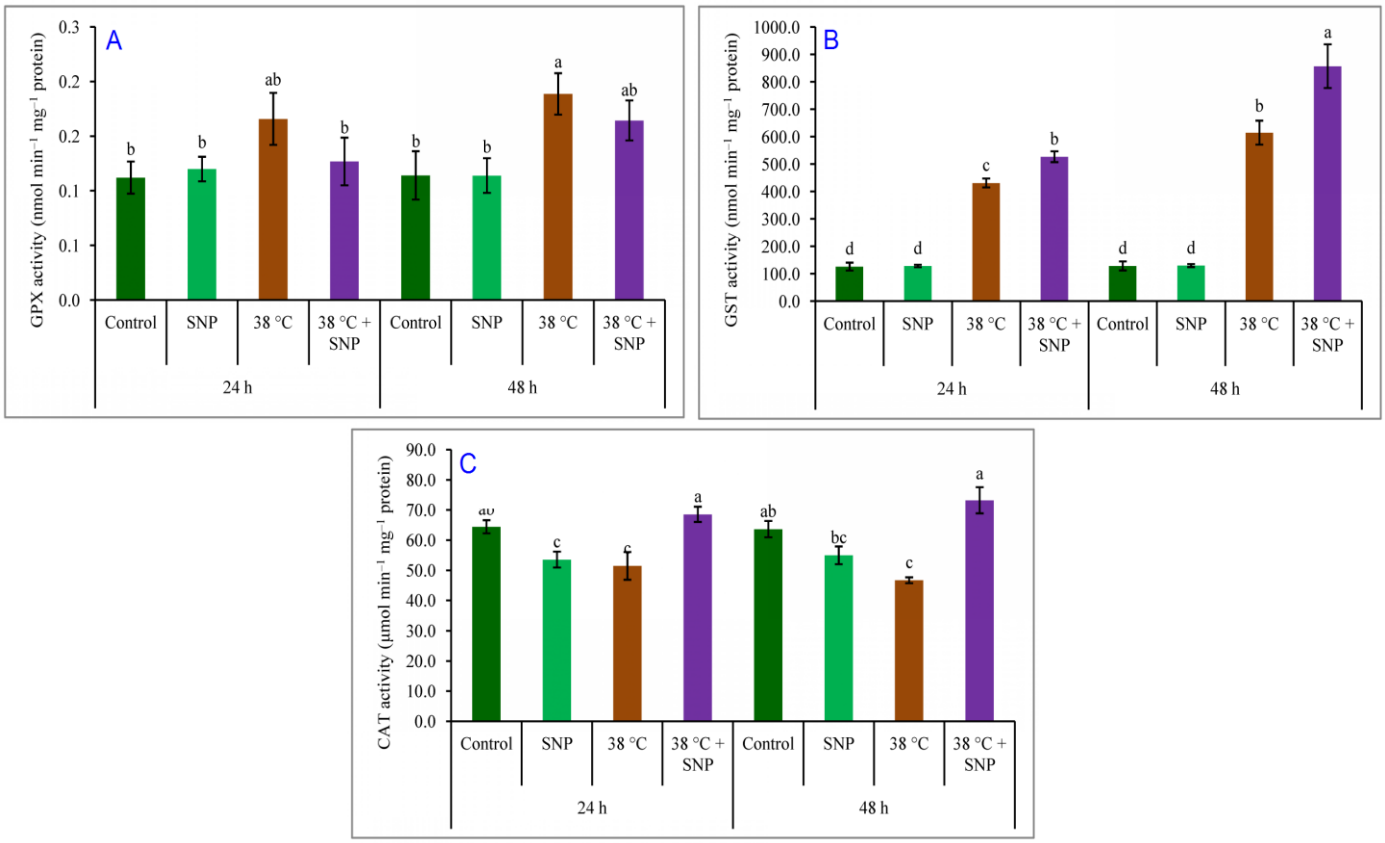

Fig 4. Activities of GPX (A), GST (B) and CAT (C) in wheat leaves induced by high temperature, SNP and their combination. Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $P \leq 0.05$ applying DMRT.

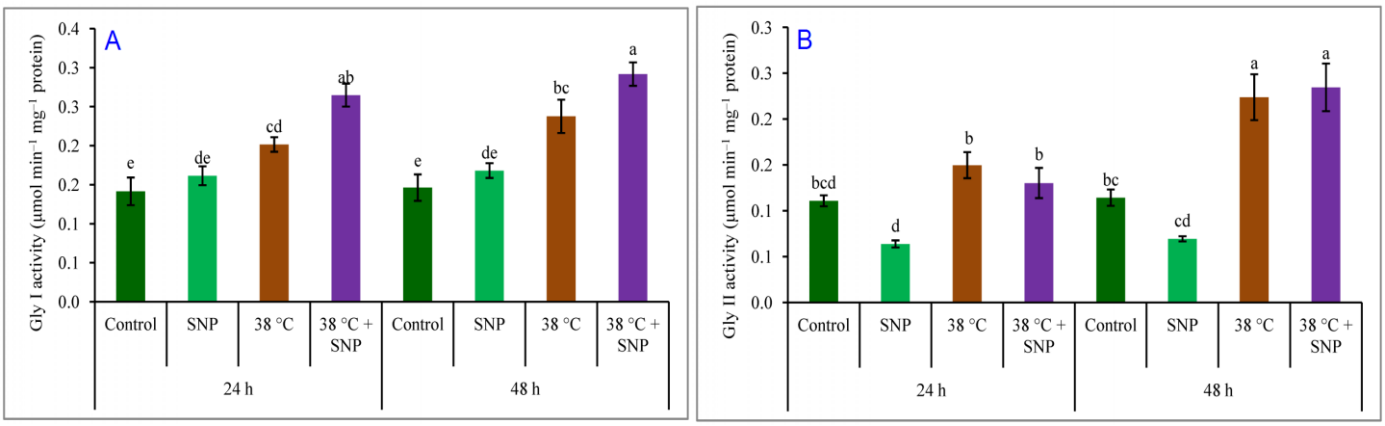

Fig 5. Activities of Gly I (A) and Gly II (B) in wheat leaves induced by high temperature, SNP and their combination. Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $P \leq 0.05$ applying DMRT.

electrophilic xenobiotic substrates with the GSH and it is associated with responses to various forms of abiotic stress and confers stress tolerance in plants (Hossain et al., 2006; Dixon et al., 2010). In our study, GST activity in wheat plants profoundly increased in response to heat stress (Fig. 4B). More importantly, heat stress seedlings supplemented with SNP also increased the activities further. This enhanced activity of GST decreased the levels of H_2O_2 and lipid peroxidation in wheat seedlings under heat stress condition (Fig. 1A, B). Similar increases in GST activity after SNP supplementation under stress were observed by other researchers (Murgia et al. 2004; Hasanuzzaman et al., 2011a). Catalases are important enzymes that convert H_2O_2 into H_2O, thus protecting the cell from the damaging effects of H_2O_2 accumulation (Sánchez-Casas and Klessig, 1994). In our study, the CAT activity significantly decreased upon exposure to any duration of heat treatment (Fig. 4C) which might be due to its inactivation by the accumulated H_2O_2 induced by water shortage, and could also be partly explained by the photoinactivation of the enzyme (Zhang and Kirkham, 1994). Our results are well agreed with the findings of other researchers (Jiang and Huang, 2001; Anjali et al., 2006; Lu et al., 2007) who observed significant reduction in CAT activity under heat stress. However, SNP supplemented seedlings grown under heat stress condition returned the CAT activity to the activity similar to control, which indicates a protective

role for exogenous NO in scavenging H_2O_2 in wheat seedlings under heat stress (Fig. 1B, 4C). Increased CAT activities in NO-supplemented plants under various forms of stress have been reported earlier (Jin et al. 2010; Xu et al., 2010; Hasanuzzaman et al., 2011a). Efficient detoxification of MG is necessary to provide protection against abiotic stress-induced damage in plants. Plants possess glyoxalase systems to protect their cells against the damaging effects of MG, which involves 2 enzymes viz. Gly I and Gly II (Yadav et al., 2005a, b). Firstly, Gly I catalyzes the isomerization of the spontaneously formed hemithioacetal adduct between GSH and 2-oxoaldehydes (such as MG) into *S*-2-hydroxyacylglutathione (Creighton and Hamilton, 2001; Thornalley, 2003). Secondly, Gly II hydrolyses these thiolesters, and, in the case of MG catabolism, produces D-lactate and GSH from SLG (vander Jagt, 1993). In our experiment, both the Gly I and Gly II activities slightly increased in the seedlings exposed to heat treatment (Fig. 5A, B). However, the SNP supplemented seedlings, on the other hand, maintained higher Gly I activity at either duration of heat stress. However, the activity of Gly II was not affected by SNP supplementation (Fig. 5A, B) which suggests that the heat-induced increase in the Gly II activity under heat stress was sufficient for this plant to detoxify excess amount of toxic MG. These results were partially supported by Hasanuzzaman et al. (2011a).

Materials and methods

Plant materials and stress treatments

Wheat (*Triticum aestivum* L. cv. Pradip) seeds of uniform size were selected and surface-sterilized with 1% sodium hypochloride solution for 10 minutes followed by washing several times with sterile distilled water. The seeds were then sown in Petri plates (9 cm) lined with 6 layers of filter paper moistened with 10 mL of distilled water for germination for two days. Germinated seedlings were then allowed to grow under controlled conditions (light, 100 µmol photon m^{-2} s^{-1}; temperature, 25±2°C; RH, 65–70%) that contained 10,000-fold diluted Hyponex solution (Hyponex, Japan). After 8 days, seedlings were grown with 0.25 mM sodium nitroprusside (SNP, $Na_2[Fe(CN)_5NO]\cdot 2H_2O$ – a NO donor) or without (control) at high temperatures at 38 °C for 24 and 48 h. Control plants were grown in Hyponex solution only. The experiment was repeated three times under the same conditions.

Determination of chlorophyll content

Chlorophyll (Chl) content was determined by taking fresh leaf samples (0.5 g) from randomly selected seedlings. The samples were homogenized with 10 mL of acetone (80% v/v) using pre-cooled pestle and mortar and the homogenate was centrifuged at 5,000× *g* for 10 min. The absorbance was measured with a UV-visible spectrophotometer at 663 and 645 nm and Chl contents were calculated using the equations proposed by Arnon (1949).

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating MDA, a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968) with slight modifications as described by Hasanuzzaman et al. (2011a).

Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu et al. (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf samples with 3 mL of 50 mM potassium-phosphate (K-P) buffer (pH 6.5) at 4° C. The homogenate was centrifuged at 11,500× *g* for 15 min. Three mL of supernatant was mixed with 1 mL of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v) and kept in room temperature for 10 min. After that the mixture was again centrifuged at 11,500× *g* for 15 min. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content (Є=0.28 $\mu M^{-1}cm^{-1}$) and expressed as µmol g^{-1} fresh weight.

Extraction and measurement of ascorbate and glutathione

Wheat leaves (0.5 g fresh weight) were homogenized in 3 mL ice-cold acidic extraction buffer (5% meta-phosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500× *g* for 15 min at 4°C and the supernatant was collected for analysis of ascorbate and glutathione. Ascorbate content was determined following the method of Huang et al. (2005) with some modifications as described by Hasanuzzaman et al. (2011a). The glutathione pool was assayed according to previously described methods (Yu et al. 2003) with modifications as described by Paradiso et al. (2008) and Hasanuzzaman et al. (2011a). Standard curves with known concentrations of GSH and GSSG were used. The content of GSH was calculated by subtracting GSSG from total GSH.

Determination of protein

The protein concentration of each sample was determined following the method of Bradford (1976) using BSA as a protein standard.

Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 mL of 50 mM ice-cold K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM *β*-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500× *g* for 10 min and the supernatants were used for determination of enzyme activity. All procedures were performed at 0−4°C. APX (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 700 µL. The reaction was started by the addition of H_2O_2 and the activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. MDHAR (EC: 1.6.5.4) activity was determined by the method of Hossain et al. (1984). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO and enzyme solution in a final volume of 700 µL. The reaction was started by the addition of AO. The activity was calculated from the change in absorbance at 340 nm for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. DHAR (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. The activity was calculated from the

change in absorbance at 265 nm for 1 min using extinction coefficient of 14 $mM^{-1}cm^{-1}$. GR (EC: 1.6.4.2) activity was measured by the method of Hasanuzzaman et al. (2011b). The reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 mL. The reaction was initiated with GSSG and the decrease in absorbance at 340 nm was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. GST (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Hossain et al. (2006) with some modifications. The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4- dinitrobenzene (CDNB), and enzyme solution in a final volume of 700 μL. The enzyme reaction was initiated by the addition of CDNB and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$. GPX (EC: 1.11.1.9) activity was measured as described by Elia et al. (2003) with slight modification as described by Hasanuzzaman et al. (2011a). CAT (EC: 1.11.1.6) activity was measured according to the method of Hasanuzzaman et al. (2011a) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction was initiated with enzyme extract and the activity was calculated using the extinction coefficient of 39.4 $M^{-1}cm^{-1}$. Glyoxalase I (EC: 4.4.1.5) assay was carried out according to Hasanuzzaman et al. (2011a). Briefly, the assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH and 3.5 mM MG in a final volume of 700 μL. The reaction was started by the addition of MG and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 $mM^{-1}cm^{-1}$. Glyoxalase II (EC: 3.1.2.6) activity was determined according to the method of Principato et al. (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM DTNB and 1 mM *S*-D - lactoylglutathione (SLG) in a final volume of 1 mL. The reaction was started by the addition of SLG and the activity was calculated using the extinction coefficient of 13.6 $mM^{-1}cm^{-1}$.

Statistical analysis

All data obtained were subjected to analysis of variance (ANOVA) and the mean differences were compared by a Duncan's multiple range test (DMRT) test using XLSTAT v.2010 software (Addinsoft 2010). Differences at $P < 0.05$ were considered significant.

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