Available online: www.notulaebiologicae.ro
Print ISSN 2067-3205; Electronic 2067-3264
Not Sci Biol, 2016, 8(2):192-198. DOI: 10.15835/nsb.8.2.9807
Notulae Scientia Biologicae
Original Article

# Protective Effect of Nitric Oxide on High Temperature Induced Oxidative Stress in Wheat (*Triticum aestivum*) Callus Culture

Hossam S. EL-BELTAGI[1]*, Osama K. AHMED[1], Adel E. HEGAZY[2]

[1]*Cairo University, Faculty of Agriculture, Biochemistry Department, PO Box 12613, Gamma St, Giza, Cairo, Egypt; lbltg@yahoo.com (*corresponding author)*
[2]*Sadat City University, Genetic Engineering and Biotechnology Institute (GEBRI), Plant Biotechnology Department, Sadat, Egypt*

**Abstract**

High temperature (HT) stress is a major environmental factor that limits plant growth, metabolism and productivity worldwide. Plant growth and development involve numerous biochemical reactions that are sensitive to temperature. Plants' responses to HT vary with the degree and duration of HT and the plants' adaptability. The protective effect of exogenous nitric oxide in alleviating high temperature induced damages of wheat (*Triticum aestivum*) callus tissues was investigated. Heat treatment (35 and 40 °C) alone or in combination with 0.5 mM sodium nitroprusside (SNP; nitric oxide donor) was applied for 72 h to callus tissues cultured on MS medium. Heat stress significantly increased lipid peroxidation, hydrogen peroxide and superoxide anion radical levels, whereas ascorbate and total glutathione contents markedly decreased. In addition, heat stress increased the activities of antioxidant defense enzymes: superoxide distumase, ascorbate peroxidase and glutathione reductase. In contrast, the addition of SNP in the culture media prevented the callus from the heat-induced oxidative damage as indicated by the decrease of MDA, $H_2O_2$ and $O_2^-$ contents, and increased the activities of antioxidant enzymes and non-enzymatic antioxidant constituents. These results provided support for the hypothesis that the exogenous applications of NO confer tolerance to high temperature stress by reducing the oxidative damage in plants.

***Keywords:*** heat stress, antioxidant enzymes, lipid peroxidation, nitric oxide, wheat

***Abbreviations:*** HT: High temperature, NO: Nitric oxide, SNP: sodium nitroprusside, MDA: malonaldehyde, $H_2O_2$: hydrogen peroxide, GSH: glutathione, AsA: Ascorbate, SOD: Superoxide dismutase, APX: ascorbate peroxidase, GR: glutathione reductase

## Introduction

Plants often experience from abiotic stress like salinity, drought, toxic metals, extreme temperatures, flooding, radiation, ozone etc. Abiotic stress is responsible for huge crop loss and reduced yield of more than 50% of some major crops (El-Beltagi *et al*., 2011a; El-Beltagi and Mohamed, 2013a; El-Beltagi *et al*., 2013; Rohbakhsh, 2013; Abdel-Hamid and Mohamed, 2014). Abiotic stresses such as extreme temperatures and low water availability frequently limit the growth and productivity of major crop species including cereals (Zhang *et al*., 2013). Future climate scenarios suggest that global warming may enhance the yield for wheat crops in some regions and reduce its productivity in zones where optimal temperatures already exist.

Global warming, however, may negatively affect wheat grain yields potentially increasing food insecurity (Hasanuzzaman *et al*., 2012a). Thus, high temperature stress is one of the important yield limiting factors in wheat. High temperature is often accompanied with low water supply, so the primary aim of cereal breeding research must be to develop cultivars tolerating both types of stresses (Tester and Bacic, 2005). Many studies on the influences of high temperatures on cereals are associated with the day- and nighttime temperatures.

The influence of high temperatures on growth and development of wheat and other crops is well documented as it damages photosynthetic membranes and causes chlorophyll loss (Mathur *et al*., 2011). There is significant linkage between the responses to heat stress and oxidative stress. Both stressed induce pathways which cause the expression and accumulation of heat shock proteins (HSP) in plants (Lee *et al*., 2000). On the other hand, there is also evidence that heating induces oxidative stress and / or expression of antioxidant enzymes in plants (Lee *et al*., 1999). Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($HO^{\cdot}$) produced in plants cause oxidative damage to cellular components, and their involvement in a number of biotic and abiotic stresses has been shown (Bowler *et al*., 1992; El-Beltagi *et al*., 2011b; Kobeasy *et al.,* 2011; El-Beltagi and Mohamed, 2013b).

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

*Received: 06 Apr 2016. Received in revised form: 15 June 2016. Accepted: 17 June 2016. Published online: 17 June 2016.*

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 highly accumulates in response to various environmental stresses (Hasanuzzaman and Fujita, 2011). Tissue damage occurs when the capacity of their antioxidative systems is insufficient to counteract the amount of ROS being generated. Malondialdehyde (MDA) is a marker commonly used for assessing membrane lipid peroxidation (Sgherri *et al*., 2003).

In plants, ROS detoxification is carried out by a network of reactions involving enzymes such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), peroxidase (POD: EC 1.11.1.7), APX (EC 1.11.1.11), glutathione reductase (GR: EC 1.6.4.2) and low-molecular weight non-enzymatic antioxidants such as ascorbate (ASC), reduced glutathione (GSH), carotinoids, ∝-tocopherol and flavonoids (Mittler, 2002). SOD removes superoxide anion free radicals accompanying with the formation of $H_2O_2$, which is then detoxified by CAT and POD. In the ascorbate–glutathione cycle, APX reduces $H_2O_2$ using ASCas an electron donor. Oxidized ASC is then reduced by GSH generated from oxidized glutathione (GSSG) catalyzed by GR at the expense of NADPH (Panchuk *et al*., 2002). In environmental stress conditions such as drought, high activities of antioxidant enzymes and high contents of nonenzymatic constituents are important for plants to tolerate stresses (Gong *et al*., 2005). Non-enzymatic antioxidants include carotenoids, ascorbate (ASC), glutathione (GSH), tocopherol, flavanones, anthocyanins etc. (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).

Nitric oxide (NO) is a signaling molecule that has been implicated in the activation of plant defenses. NO is a bioactive free radical which plays important roles in many physiological processes in plants, such as growth, development, senescence and adaptive responses to multiple stresses (Zhao *et al*., 2004; Graziano and Lamattina, 2005; Shehab *et al*., 2010; El-Beltagi *et al*., 2015). Under ROS-related toxicity, NO may act as a chain breaker and thus limits the oxidative damage. Recently, a function of NO has been reported to protect plants against oxidative stress under various adverse conditions (Beligni and Lamattina, 2002; Shi *et al*., 2005). Many previous studies have reported the presence of NO in the plant kingdom and its involvement in growth, development and defense responses (Beligni and Lamattina, 1999; Zhang *et al*., 2003).

Considering the severe effects of high temperatures, developing thermo tolerance 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 within whole plant as well as at molecular level (Tiroli-Cepeda and Ramos, 2010). In the present study it was evaluated the effects of sodium nitroprusside (SNP, a nitric oxide donor) *in vitro* treatment on wheat (*Triticum aestivum*) callus on enzymatic and non-enzymatic antioxidant systems under heat stress condition.

## Materials and Methods

*Plant materials and surface sterilization*

All excised tissues of young steams of wheat were collected from Agriculture Research Center (ARC), Giza governorate, and stored temporarily in an antioxidant solution (150 mg/l citric acid and 100 mg/l ascorbic acid), prior to surface-sterilization in 1% chlorox for 20 min, 2 drops/ 100 ml solution of Tween 20 as wetting agent, then rinsed three times with sterile distilled water.

*Callus initiation and maintenance*

Callus was initiated from the young sterilized stems of wheat. The callus was initiated in a MS-Medium (Murashige and Skoog, 1962) supplemented with 3.0 mg/L Naphthalene acetic acid (NAA) and 2.5 mg/L Benzyladenine (BA), 20 g/L sucrose and 8 g/L agar. The medium was adjusted to pH 5.8 and then sterilized at 121 °C for 20 min, incubated in dark at 25 °C. After three weeks, the initiated callus tissues were transferred to fresh medium, and sub cultured every four weeks.

*Heat and chemical treatments*

The experimental design consisted of callus grown with 0.25 mM sodium nitroprusside (SNP, $Na_2$ $[Fe(CN)_5NO]\cdot 2H_2O$ – a NO donor) or without (control) at different temperatures at 25, 35, 40 °C (Hasanuzzaman *et al*., 2012b). Callus cells were harvested after four weeks, washed twice with 100 ml distilled water on a porous – glass funnel with filter paper (Whatman No.1) and the excess water was removed by filtration; callus cells were further frozen in liquid nitrogen and stored in the deep freezer until further analysis.

*Determination of ascorbic acid and total soluble sugars*

Ascorbate content was measured using 2, 4-dinitrophenolindophenol. The absorbance was measured at 520 nm according to Omaye *et al*. (1979). Total soluble sugars were determined in the callus ethanolic extract using phenol-sulfuric acid method according to Dubois *et al*. (1956).

*Analysis of lipid peroxidation*

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content following the method of Heath and Packer (1968) modified by Song *et al*. (2006). Callus weight (0.5 g) was homogenized using mortar and pestle in trichloroacetic acid (10%) and then the homogenate was centrifuged at 4000 x g for 30 min. Supernatant (2 mL aliquot) was mixed with 2 mL of trichloroacetic acid (10%) containing thiobarbituric acid (0.5%). The mixture was heated at 100 °C for 30 min. The absorbance of the supernatant was measured at 532 nm, with a reading at 600 nm subtracted from it to account for non-specific turbidity. The amount of malonaldehyde was calculated using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

*Hydrogen peroxide determination*

The hydrogen peroxide content in the callus was assayed according to the method of Velikova *et al*. (2000). Callus was crushed into fine powder using liquid nitrogen then homogenized with TCA (0.1% w/v). The extract was centrifuged at 12,000 x g for 15 min, then the supernatant (0.5 Ml) was added to 0.5 ml of potassium phosphate buffer pH 7.0 (10 mM) and 1 ml of 1 M KI, and the absorbance was read at 390 nm. The content of $H_2O_2$ was given on a standard curve.

*The production rate of $O_2^{\cdot-}$*

The production rate of $O_2^{\cdot-}$ was measured by a modified method as described by Elstner and Heupel (1976). Fresh mass (200 mg) from culture was homogenized in 1 ml of 50 mM phosphate buffer (pH 7.8) and the homogenate was centrifuged at 10,000 x g for 10 min. Then 0.5 ml of the supernatant was added to 0.5 ml 50 mM phosphate buffer (pH 7.8) and 0.1 ml of 10 mM hydroxylamine hydrochloride. After 1 h reaction at 25 °C, the mixture was supplemented with 1 ml of 17 mM sulfanilamide and 1 ml 7 mM α-naphthylamine, kept at 25 °C for 20 min; the specific absorbance was determined at 530 nm. Sodium nitrite was used as standard solution to calculate the production rate of $O_2^{\cdot-}$.

*Total glutathione determination*

The level of total glutathione (GSH) was determined with Ellman's reagent according to De Vos *et al.* (1992). The buffer was mixed with $K_2HPO_4$ (630 µl) of 0.5 M and 25 µl of mM 5, 5'–dithiobis (2-nitrobenzoic acid) (final pH = 7). The absorbance at 412 nm was read after 2 min. GSH was used as a standard.

*Assay of total soluble protein*

Soluble proteins were measured by the Bio-Rad micro assay modification of the Bradford (1976) procedure, using crystalline bovine serum albumin as a reference.

*Preparation of enzyme extracts*

The callus samples (1.0 g) were crushed into fine powder using liquid nitrogen. Soluble protein was extracted by homogenizing the powder in 5 mL 50 mM phosphate buffer (pH 7.8) containing ethylenediamine-*N*,*N*,*N*,*N*-tetraacetic acid (EDTA, 1 mM) and polyvinylpyrrilidone (PVP, 1%) in addition to ASC (1 mM) in the case of APX assay. The samples' homogenate was centrifuged at 15,000 x g for 20 min, and the supernatant was used for the following enzyme activity assays.

*Antioxidant enzymes activity*

Superoxide dismutase (SOD) (EC 1.15.1.1) was assayed by measuring the ability of enzyme preparation to inhibit the photochemical reduction of NBT (Beauchamp and Fridovich, 1971). The reaction mixture (3 ml) contained phosphate buffer pH 7.8 (50 Mm), methionine (13 mM), nitroblue tetrazolium (NBT, 75 µM), EDTA (1.0 mM) and enzyme extract (20 µL). Riboflavin (2 µM) was added last and the reaction was initiated by placing the tubes 30 cm below 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with black cloth. Non-illuminated tubes served as control. The absorbance at 560 nm was recorded. One unit of SOD was defined as that being contained in the volume of extract that caused a 50% inhibition of the SOD-inhibitable fraction of the NBT reduction.

Ascorbate peroxidase activity was determined spectrophotometrically by following the decrease in the absorbance at 290 nm ($E = 2.8\ mM^{-1}\ cm^{-1}$) using the method of Nakano and Asada (1981). The reaction mixture (3 mL) contained potassium phosphate buffer pH 7.0 (50 mM), ASC (0.3 mM), $H_2O_2$ (0.1 mM) and enzyme extract (50 µL). The rates were corrected for non-enzymatic oxidation of ASC by the inclusion of reaction mixture without enzyme extract. One unit was defined as reduction of 0.01 in absorbance at 290 nm per 1 min/ mgprotein.

Glutathione reductase activity (GR; EC 1.6.4.2.) was determined based on the decrease in absorbance at 340 nm due to the oxidation of NADPH to NADP according to the method of Foyer and Halliwell (1976), with minor modifications. The reaction mixture (3 mL) consisted of 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 0.5 mM GSSG, 0.2 mM NADPH and 0.1 mL enzyme extract. The reaction was started by the addition of GSSG and the NADPH oxidation rate was monitored at 340 nm for 3 min. Enzyme activity was determined using the molar extinction coefficient for NADPH ($6.2\ mM^{-1}\ cm^{-1}$).

*Statistical analysis*

The data were analyzed using SPSS (version 10) program. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. P-values < 0.05 were considered significant.

## Results and Discussion

*Effects of heat stress and SNP treatments on $H_2O_2$ concentrations, lipid peroxidation and superoxide anion ($O_2^{\cdot-}$) production rate*

Since oxidative damage is generated under heat stress through the formation of ROS in plants, $H_2O_2$ was measured. It is a main kind of active oxygen specie that accumulates under heat stress (Dat *et al.*, 1998). Compared to control, high temperature stress significantly increased $H_2O_2$ contents in wheat callus (Table 1). The $H_2O_2$ contents increased with increasing the high temperature stress, resulting in a significantly higher value under 40 °C treatments. The $H_2O_2$ contents increased in a temperature dependent manner. Addition of 0.25 mM SNP at temperature of 35 and 40 °C in the culture media significantly decreased the accumulation of $H_2O_2$ in wheat callus under high temperature stress.

Lipid peroxidation is one of the consequences of oxidative damage and significantly increased with increasing temperatures as determined by the accumulation of MDA. As shown in Table 1, heat stress significantly increased MDA concentration in wheat callus compared to the control in a trend similar to $H_2O_2$ change. The MDA content recorded the highest accumulation in the heat stressed callus at 40 °C compared to the negative control. The application of NO donor significantly reduced the accumulation of MDA, especially at high temperature stress conditions. Increased $H_2O_2$ and lipid peroxidation have been observed in plants growing under different stress conditions (Sun *et al.*, 2007; Hasanuzzaman *et al.*, 2013).

Moreover, a significant ($P < 0.05$) increase in the production rate of $O_2^{\cdot-}$ was observed in heated samples and this increase was more pronounced at 40 °C (3.15 ± 0.18 µmol/ min g FW) compared to control (0.74 ± 0.07 µmol/ min g FW). Xu *et al.* (2006) reported that heat stress caused a considerable significant increase in the $H_2O_2$ and $O_2^{\cdot-}$ values of in two turf grass species leaves exposed to three heat stress levels (38, 42, 46 °C) for 14 h, respectively. Heat stress could lead to an accumulation of free radicals such as $O_2^{\cdot-}$ and $H_2O_2$ which unbalance the system of free radical elimination and thus induce lipid peroxidation. Addition of 0.25 mM SNP at temperature of 35 and 40 °C in the culture media significantly decreased the accumulation of $O_2^{\cdot-}$ in wheat callus under high temperature stress (Table1).

Table 1. Effects of heat stress and SNP treatments on the $H_2O_2$ contents, lipid peroxidation (MDA) and superoxide anion radical ($O_2^{\cdot-}$) in wheat callus

| $O_2^{\cdot-}$ (μ mol/ min g FW) | MDA (μ mol/ g FW) | $H_2O_2$ (μ mol/g FW) | Treatments |
|---|---|---|---|
| 0.74 ± 0.07$^{f}$ | 2.66 ± 0.42$^{d}$ | 39.52 ± 1.72$^{e}$ | Negative control |
| 0.93 ± 0.05$^{e}$ | 2.79 ± 0.56$^{d}$ | 32.06 ± 1.33$^{f}$ | SNP |
| 1.98 ± 0.11$^{b}$ | 19.73 ± 1.05$^{b}$ | 95.02 ± 4.39$^{b}$ | Temperature (35 °C) |
| 1.18 ± 0.06$^{d}$ | 14.63 ± 2.08$^{c}$ | 62.41 ± 2.23$^{d}$ | Temperature (35 °C) + SNP |
| 3.15 ± 0.18$^{a}$ | 27.59 ± 2.82$^{a}$ | 107.62 ± 2.35$^{a}$ | Temperature (40 °C) |
| 1.85 ± 0.13$^{c}$ | 19.32 ± 1.41$^{b}$ | 88.02 ± 5.41$^{c}$ | Temperature (40 °C) + SNP |
| 0.12 | 2.89 | 5.80 | LSD |

Means in the same column with the same letter were not significantly at P < 0.05.

Table 2. Effects of heat stress and SNP treatments on the content of reduced glutathione, total ascorbic acid and total soluble sugars in wheat callus

| Total soluble sugars (mg/g FW) | Total AsA (mg/g FW) | GSH (μmol/g FW) | Treatments |
|---|---|---|---|
| 8.66 ± 0.20$^{f}$ | 9.55 ± 0.31$^{b}$ | 6.78 ± 0.47$^{b}$ | Negative control |
| 10.48 ± 0.48$^{e}$ | 10.21 ± 0.30$^{a}$ | 7.68 ± 0.35$^{a}$ | SNP |
| 13.01 ± 0.46$^{d}$ | 6.84 ± 0.28$^{c}$ | 5.37 ± 0.49$^{c}$ | Temperature (35 °C) |
| 15.44 ± 0.39$^{b}$ | 7.26 ± 0.49$^{c}$ | 6.57 ± 0.53$^{b}$ | Temperature (35 °C) + SNP |
| 14.43 ± 0.31$^{c}$ | 5.52 ± 0.39$^{e}$ | 3.29 ± 0.38$^{e}$ | Temperature (40 °C) |
| 17.13 ± 0.46$^{a}$ | 6.30 ± 0.22$^{d}$ | 4.70 ± 0.64$^{d}$ | Temperature (40 °C) + SNP |
| 0.56 | 0.43 | 0.35 | LSD |

Means in the same column with the same letter were not significantly at P< 0.05.

Table 3. Effects of heat stress and SNP treatments on the activities of SOD, APX and GR in wheat callus

| GR activity (μmol/mg protein/min) | APX activity (Unit/mg protein/min) | SOD activity (Unit/mg protein/min) | Treatments |
|---|---|---|---|
| 4.38 ± 0.26$^{e}$ | 17.23 ± 0.17$^{d}$ | 206.15 ± 1.61$^{f}$ | Negative control |
| 5.23 ± 0.30$^{d}$ | 21.11 ± 0.23$^{c}$ | 213.39 ± 2.60$^{e}$ | SNP |
| 7.82 ± 0.40$^{c}$ | 23.76 ± 0.04$^{b}$ | 227.42 ± 1.80$^{d}$ | Temperature (35 °C) |
| 9.81 ± 0.57$^{b}$ | 27.21 ± 0.33$^{a}$ | 242.85 ± 2.19$^{b}$ | Temperature (35 °C) + SNP |
| 9.62 ± 0.38$^{b}$ | 24.14 ± 0.32$^{b}$ | 236.39 ± 2.61$^{c}$ | Temperature (40 °C) |
| 11.83 ± 0.2$^{a}$ | 27.93 ± 0.21$^{a}$ | 252.35 ± 2.33$^{a}$ | Temperature (40 °C) + SNP |
| 0.43 | 0.64 | 2.93 | LSD |

Means in the same column with the same letter were not significantly at P< 0.05.

Results also revealed that the heat stress treatment induced oxidative stress in wheat callus as indicated by the increased accumulation of $H_2O_2$ and MAD. The increased $H_2O_2$ concentration in the callus growing under heat stress probably resulted from abiotic stress-induced increase in the rate of $O_2^{\cdot-}$ production, which is considered to be the main precursor of mitochondrial $H_2O_2$ (Mittler, 2002; Yin *et al*., 2008).

High temperature stress disrupts membrane permeability is influenced by peroxidation of the lipid membrane (Mandhania *et al*., 2006). Membrane injury under high temperature is related to an increased production of highly toxic reactive oxygen species. Lipid peroxidation was measured as the amount of produced MDA when polyunsaturated fatty acids in the membrane undergo oxidation by the accumulation of free oxygen radicals. Lipid peroxidation is ascribed to oxidative damage and is often used as an indicator of degree of damage (Bor *et al*., 2003).

In addition, the SNP supplemented heat-stressed seedlings maintained significantly lower levels of $H_2O_2$ content, as compared to the seedlings subjected to heat stress without SNP (Hasanuzzaman *et al*., 2012b). This indicated 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*., 2011; Kong *et al*., 2011). Zottini *et al*. (2002) have reported previously that NO can affect plant mitochondrial functionality. The currentr results showed that oxidative stress in wheat callus induced by heat stress was effectively alleviated by the application of SNP as a NO donor.

*Glutathione and ascorbate contents*

The levels of the most abundant soluble non-enzymatic antioxidants, GSH and AsA, were determined to establish their roles at high temperature stress in 4 week old wheat callus cultures. After 4 weeks, GSH content significantly decreased under heat stress (Table 2). It reached the lowest content at the highest temperature stress in respect to the negative control. The treatment by low concentration of SNP led to an increase of GSH content in respect to the control.

In addition, high temperature stress significantly decreased the levels of AsA in wheat callus compared to untreated controls. Treatment with SNP caused an obvious increase in their levels, even under heat stress, suggesting a protective role of NO against the oxidative stress. The hereby results are in agreement with those of Hasanuzzaman *et al*. (2012b), who found that there were marked decreases in AsA contents in response to heat stress, as compared to the control. Heat-stressed seedlings supplemented with SNP had significantly higher AsA content at any duration, as compared to seedlings subjected to heat stress without SNP.

Drastic increases in GSH contents were observed in response to heat stress, as compared to the control. The seedlings exposed to 24 and 48 h of heat stress showed 90% and 153% increase in reduced glutathione (GSH) content compared to their respective control.

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 the removal of ROS, MG and other endogenous toxic compounds (Noctor *et al*., 2012). Results demonstrated that AsA and GSH contents were significantly decreased by heat treatment, but the SNP administration increased the contents of ASA and glutathione under high temperature stress condition.

*Total soluble sugars*

High temperature stress is also known to affect the metabolism of soluble sugars, a group of compounds that may act as compatible solutes. As shown in Table 2, heat stress and SNP treatments increased the soluble sugars. Total soluble sugar content increased rapidly and reached its maximum at the highest temperature of 40 °C and SNP treatments. The current results agreed with previous results which found that SNP treatments under stress reflected a highly significant increase in the total soluble sugars in rice plants (Shehab *et al*., 2010). It has been suggested that under stress, soluble sugars function as osmolytes to maintain and stabilize cell proteins and structures during stress (Bohnert *et al*., 1995). Several genes that are involved in the metabolism of these osmolytes have been found to increase the tolerance of abiotic stress in plants (Bartels and Sunkar, 2005).

*Responses of antioxidant enzymes to SNP treatments*

As shown in Table 3, the activity of the antioxidant enzymes significantly increased under the high temperature stress condition and the increase in the activity was temperature-dependent. The results indicated that the growing of callus under heat stress condition (35 and 40 °C) led to a significantly increase in SOD, APX and GR activity. In addition, further enhancement of SOD, APX and GR activity was observed by the addition of 0.25 mM SNP to the culture media exposed to heat stress and compared to heat stress alone. The treatment by low concentration of NO improved the activity of antioxidant enzymes SOD, APX and GR.

Increases in ROS scavenging enzymes activity in plants have been observed under different environmental stresses, for example increase in ROS-scavenging enzymes increased under heat stress in the callus of reed and wheat seedlings (Song *et al*., 2006; Hasanuzzaman *et al*., 2012b). The hereby obtained results showed that activities of SOD, APX and GR in wheat callus were greatly induced under the high temperature stress and the addition of SNP significantly enhanced the activity of these ROS-scavenging enzymes, indicating that SNP can play an important role in protecting the plants from the oxidative damage caused by high temperature stress, by inducing the antioxidant enzymes activities.

SOD is a major scavenger of $O_2^{\cdot-}$, whose enzymatic action results in the formation of $H_2O_2$. CAT, APX, POD are all related to remove of $H_2O_2$. It was likely that high temperature activated these antioxidant enzymes, whose cooperation with each other contributed to scavenging of $H_2O_2$ generated by oxidation stress and alleviated oxidative injury imposed by heat stress in wheat callus.

The results obtained hereby are in agreement with those of Song *et al*. (2006) who suggested that NO can effectively protect calluses of two ecotypes of reed from oxidative stress induced by heat and that NO might act as a signal in activating active oxygen scavenging enzymes under heat stress and thus confer reed callus thermo tolerance.

## Conclusions

In conclusion, heat stress had adverse effects on the growth attributes of wheat. It generated reactive oxygen species (ROS), which lead to oxidative stress. However, exogenous application of NO played a protective role, by effectively scavenging reactive oxygen species through increasing the content of activities of non-enzymatic antioxidant such as AsA, GSH and antioxidant enzymes like SOD, APX and GR. High accumulation of total soluble sugars by exogenous application of NO regulated osmoregulation in the wheat plants under heat stress.

## Acknowledgements

The authors extend their appreciation to the Faculty of Agriculture, Cairo University and to Genetic Engineering and Biotechnology Institute, Sadat City University, Egypt, for funding the work.

## References

Abdel-Hamid AME, Mohamed HI (2014). The effect of the exogenous gibberllic acid on two salt stressed barley cultivars. European Scientific Journal 10(6):228-245.

Bartels D, Sunkar R (2005). Drought and salt tolerance in plants. Critical Reviews in Plant Science 24:23-58.

Beauchamp C, Fridovich I (1971). Superoxide dismutase: improved assays and assay applicable to acrylamide gels. Analytical Biochemistry 44(1):276-287.

Beligni MV, Lamattina L (1999). Nitric oxide counteracts cytotoxic processes mediated by reactive oxygen species in plant tissues. Planta 208(3):337-344.

Beligni MV, Lamattina L (2002). Nitric oxide interferes with plant photo-oxidative stress by detoxifying reactive oxygen species. Plant, Cell & Environment 25(6):737-748.

Bohnert HJ, Nelson DE, Jensen RG (1995). Adaptations to environmental stresses. Plant Cell 7(7):1099-1111.

Bor M, Ozdemir F, Turkan I (2003). The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. The Plant Science 164(1):77-84.

Bowler C, Montagu MV, Inze D (1992). Superoxide dismutase and stress tolerance. Annual Review of Plant Biology 43(1):83-116.

Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72(1-2):248-254.

Dat JF, Lopez-Delgado H, Foyer CH, Scott IM (1998). Parallel changes in $H_2O_2$ and catalase during thermo tolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiology 116(4):1351-1357.

De Vos CH, Vonk MJ, Vooijs R, Henk S (1992). Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucbalus*. Plant Physiology 98(3):853-858.

Dubois M, Smith F, Gilles KA, Hamilton JK, Rebers PA (1956). Colourimetric method for determination of sugars and related substances. Analytical Biochemistry 28(3):350-356.

El-Beltagi HS, Ahmed OK, El-Desouky W (2011a). Effect of low doses γ-irradiation on oxidative stress and secondary metabolites production of rosemary *(Rosmarinus officinalis* L.) callus culture. Radiation Physics and Chemistry 80(9):968-976.

El-Beltagi HS, Kesba HH, Abdel-Alim AI, Al-Sayed AA (2011b). Effect of root-knot nematode and two species of crown on antioxidant activity of grape leaves. African Journal of Biotechnology 10(57):12202-12210.

El-Beltagi HS, Mohamed HI (2013a). Alleviation of cadmium toxicity in *Pisum sativum* L. seedlings by calcium chloride. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 41(1):157-168.

El-Beltagi HS, Mohamed HI (2013b). Reactive oxygen species, lipid peroxidation and antioxidative defense mechanism. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 41(1):44-57.

El-Beltagi HS, Mohamed HI, Mohammed AMA, Zaki LM, Mogazy AM (2013). Physiological and biochemical effects of γ-irradiation on cowpea plants (*Vigna sinensis*) under salt stress. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 41(1):104-114.

El-Beltagi HS, Ahmed OK, Hegazy AE (2015). Molecular role of nitric oxide in secondary products production in *Ginkgo biloba* cell suspension culture. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43(1):12-18.

Elstner EF, Heupel A (1976). Inhibition of nitrite formation from hydroxylaminonium- chloride: a simple assay for superoxide dismutase. Analytical Biochemistry 70:616-620.

Foyer CH, Halliwell B (1976). The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133(1):21-25.

Gill SS, Tuteja N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48(12):909-930.

Gong H Zhu X, Chen K, Wang S, Zhang C (2005). Silicon alleviates oxidative damage of wheat plants in pots under drought. Plant Science 169(2):313-321.

Graziano M, Lamattina L (2005). Nitric oxide and iron in plants: an emerging and converging story. Trends in plant Science 10(1):4-8.

Hasanuzzaman M, Fujita M (2011). Selenium pretreatment upregulates the antioxidant defense and methylglyoxal detoxification system and confers enhanced tolerance to drought stress in rapeseed seedlings. Biological Trace Element Research 143(3):1758-1776.

Hasanuzzaman M, Hossain MA, Fujita M (2011). Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings. Plant Biotechnology Reports 5(4):353-365.

Hasanuzzaman M, Hossain MA, da Silva JAT, Fujita M (2012a). Plant responses and tolerance to abiotic oxidative stress: Antioxidant defenses is a key factors. In: Bandi V, Shanker AK, Shanker C, Mandapaka M (Eds). Crop stress and its management: Perspectives and strategies. Springer, Berlin pp 261-316.

Hasanuzzaman M, Nahar K, Mahabub A, Fujita M (2012b). Exogenous nitric oxide alleviates high temperature induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by modulating the antioxidant defense and glyoxalase system. Australian Journal of Crop Science 6(8):1314-1323.

Hasanuzzaman M, Nahar K, Mahabub A, Roychowdhury R, Fujita M (2013). Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. International Journal of Molecular Sciences 14(5):9643-9684.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics 125(1):189-98.

Kim K, Portis J (2004). Oxygen-dependent $H_2O_2$ production by Rubisco. FEBS Letters 571(1):124-128.

Kong W, Huang C, Chen Q, Zou Y, Zhang J (2011). Nitric oxide alleviates heat stress-induced oxidative damage in *Pleurotus eryngii* var. tuoliensis. Fungal Genetics and Biology 49(1):15-20.

Kobeasy MI, El-Beltagi HS, El-Shazly MA, Khattab EAH (2011). Induction of resistance in *Arachis hypogaea* L. against Peanut Mottle Virus by nitric oxide and salicylic acid. Physiological and Molecular Plant Pathology 76(2):112-118.

Lee HS, Kim KY, You SH, Kwon SY, Kwak SS (1999). Molecular characterization and expression of a cDNA encoding copper/zinc superoxide dismutase from cultured cells of cassava (*Manihot esculenta* Crantz). Molecular and General Genetics 262(4-5):807-814.

Lee BH Won SH, Lee HS, Miyao M, Chung WI, Kim IJ, Jo J (2000). Expression of the chloroplastlocalized small heat shock protein by oxidative stress in rice. Gene 245(2):283-290.

Mandhania S, Madan S, Sawhney V (2006). Antioxidant defense mechanism under salt stress in wheatseedlings. Biologia Plantarum 227(2):227-231.

Mathur S, Allakhverdiev SI, Jajoo A (2011). Analysis of high temperature stress on the dynamics of antenna size and reducing side heterogeneity of Photosystem II in wheat leaves (*Triticum aestivum*). Biochimica et Biophysica Acta (BBA)- Bioenergetics 1807(1):22-29.

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7(9):405-410.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15:473-497.

Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant and Cell Physiology 22(5):867-880.

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G, Foyer CH (2012). Glutathione in plants: an integrated overview. Plant, Cell & Environment 35(2):454-484.

Omaye ST, Turnbm JD, Saubermch HE (1979). Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. Methods in Enzymology, Academic Press, New York 62:1-13.

Panchuk II, Volkov RA, Schöffl F (2002). Heat stress and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in arabidopsis. Plant Physiology 129(2):838-853.

Rohbakhsh H (2013). Alleviating adverse effects of water stress on growth and yield of forage sorghum by potassium application. Advances in Environmental Biology 7(1):40-46.

Sgherri C, Cosi E, Navari-Izzo F (2003). Phenols and antioxidative status of *Raphanus sativus* grown in copper excess. Physiologia Plantarum 118(1):21-28.

Shehab GMG, Ahmed OK, El-Beltagi HS (2010). Nitric oxide treatment alleviates drought stress in rice plants (*Oryza sativa*). Notulae Botanicae Horti Agrobotanici Cluj-Napoca 38(1):139-148.

Shi SY, Wang G, Wang YD, Zhang LG, Zhang LX (2005). Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. Nitric Oxide 13(1):1-9.

Song L, Ding W, Zhao M, Sun B, Zhang L (2006). Nitric oxide protects against oxidative stress under heat stress in the calluses from two ecotypes of reed. Plant Science 171(1):449-458.

Sun B, Jing Y, Chen K, Song L, Chen F, Zhang L (2007). Protective effect of nitric oxide on iron deficiency-induced oxidative stress in maize (*Zea mays*). Journal of Plant Physiology 164(5):536-543.

Tester M, Bacic A (2005). Abiotic stress tolerance in grasses. From model plants to crop plants. Plant Physiology 137(3):791-793.

Tiroli-Cepeda AO, Ramos CHI (2010). Heat causes oligomeric disassembly and increases the chaperone activity of small heat shock proteins from sugarcane. Plant Physiology and Biochemistry 48(2):108-116.

Velikova V, Yordanov I, Edreva A (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants-protective role of exogenous polyamines. Plant Science 151(1):59-66.

Xu S, Li J, Zhang X, Wei H, Cui L (2006). Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. Environmental and Experimental Botany 56(3):274-285.

Yadav SK, Singla-Pareek SL, Ray M, Reddy MK, Sopory SK (2005a). Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. Biochemical and Biophysical Research Communications 337(1):61-67.

Yadav SK, Singla-Pareek SL, Reddy MK, Sopory SK (2005b). Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress. FEBS Letter 579(27):6265-6271.

Yin H, Chen QM, Yi MF (2008). Effects of short-term heat stress on oxidative damage and responses of antioxidant system in *Lilium longiflorum*. Plant Growth Regulation 54(1):45-54.

Zhang H, Shen WB, Xu LL (2003). Effects of nitric oxide on the germination of wheat seeds and its reactive oxygen species metabolisms under osmotic stress. Acta Botanica Sinica 45(8):901-905.

Zhang X, Cai J, Wollenweber B, Liu F, Dai T, Cao W, Jiang D (2013). Multiple heat anddrought events affect grain yield and accumulations of high molecular weight glutenin subunits and glutenin macropolymers in wheat. Journal of Cereal Science 57(1):134-140.

Zhao LQ, Zhang F, Guo JK, Yang YL, Li BB, Zhang LX (2004). Nitric oxide functions as a signal in salt resistance in the calluses from two ecotype of reed. Plant Physiology 134(2):845-857.

Zottini M, Formentin E, Scattolin M, Carimi F, Schiavo FL, Terzi M (2002). Nitric oxide affects plant mitochondrial functionality in vivo. FEBS Letters 515(1):75-78.