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RESEARCH ARTICLE

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Sodium nitroprusside and indole acetic acid improve the tolerance of tomato plants to heat stress by protecting against DNA damage

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ABSTRACT

Climate change represents a major threat to agriculture. High ambient temperatures, as a result of global warming, are currently limiting plant growth and development. The aim of the present study was to investigate the effect of sodium nitroprusside (SNP) in combination with indole acetic acid (IAA) on tomato (*Lycopersicon esculentum* Mill.) plants under heat stress (HS) and non-heat stress (non-HS) conditions. HS is suggested to induce the formation of reactive oxygen species, such as superoxide and hydrogen peroxide, which may lead to genotoxicity by damaging DNA, which can be detected by the comet assay (single-cell gel electrophoresis). HS substantially enhanced proline (Pro), malondialdehyde accumulation, electrolyte leakage (EL), growth reduction, and reduced physiological and biochemical parameters. However, the co-application of SNP and IAA alleviated the adverse effects of HS by promoting catalase, peroxidase, and superoxide dismutase activities and enhancing the accumulation of photosynthetic pigments (chlorophyll *a* and *b*) and Pro with a concomitant decrease in H_2O_2 and O_2^- content, EL, and DNA damage. Conversely, the treatment of tomato plants with the NO scavenger cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] along with SNP and IAA further reduced the SNP signal. Therefore, these results suggest that the application of SNP with IAA improves plant defense mechanisms against HS.

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Heat stress; DNA damage; reactive oxygen species; temperature; nitric oxide; indole acetic acid

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the most widely cultivated vegetable crop in the world. It is the second most consumed vegetable after potato, and its global per-capita consumption is 20 kg per year (Garmin 2014). Tomato production is limited because it is highly susceptible to heat stress (HS). High day-time temperature and, particularly, high night-time temperature affect tomato productivity (Moore & Thomas 1952). HS leads to poor pollen formation and loss of pollen viability owing to the disruption of carbohydrate metabolism, proline translocation, and hormonal imbalance in plants, leading to a decrease in productivity and fruit quality (Sangu et al. 2015). Many studies on the effects of HS on tomato plants (Rivero et al. 2001) and its mitigation (Suzuki et al. 2016) have been performed. In the present study, we investigated the roles of sodium nitroprusside (SNP), a nitric oxide (NO) donor, and indole acetic acid (IAA) in alleviating the adverse effects of HS on tomato plants.

Plants are sessile organisms that are constantly exposed to a wide range of fluctuating temperatures throughout the world. Variations in temperature provide an external signal to plants, permitting them to develop adaptions in order to maintain normal growth and development. Due to the phenomenon of global warming and climate changes, a rise in ambient temperature beyond a threshold level is a major cause of concern for HS to the agricultural crops (Lavania et al. 2015). As a result of high ambient temperatures, HS causes irreversible damage to the productivity of agricultural crops (Wahid et al. 2007; Lavania et al. 2015), because plants survive or grow best within a specific temperature range. A rise in the Earth's surface temperature resulting from the emission of greenhouse gasses from anthropogenic activities may further increase if the human population and global economy continue to increase at their current rates (Sánchez et al. 2014). HS induces various morpho-anatomical, physiological, biochemical, and molecular changes in plants, leading to a catastrophic loss of crop productivity. The effects of HS begin at seed germination and last until crop maturity. An increase in temperature beyond the threshold level affects seed germination and seed vigor due to thermal injury or seed death (Grass & Burris 1995). HS leads to a wide range of changes in plants at the morpho-physiological (scorching of leaves and stems, leaf senescence and abscission, inhibition of shoot and root growth, reduction in flower number, pollen tube growth and release, pollen infertility, and fruit damage) (Teixeira et al. 2013; Siddiqui et al. 2015) and anatomical levels (reduction in cell size, alteration in thylakoid organization, and stomatal closure) (Wahid et al. 2007). HS consequently affects photosynthesis, respiration, water relations, and membrane stability, and disrupts hormonal balance, and primary and secondary metabolites in plants (Wahid et al. 2007; Hemantaranjan et al. 2014). HS alters protein stability, membrane integrity, RNA species, and the activity of enzymes in chloroplasts and mitochondria, resulting in an imbalance in metabolic homeostasis (Mittler et al. 2012; Hemantaranjan et al. 2014). An imbalance in the production of reactive oxygen species (ROS) due to abiotic stress disturbs

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metabolic homeostasis in plants, and thus autocatalytic peroxidation of membrane lipids and pigments (Wahid et al. 2007; Mittler et al. 2012). However, under HS, plants develop non-enzymatic and enzymatic detoxifying systems to maintain metabolic homeostasis and reprogram their transcriptome, proteome, metabolome, and lipidome, thereby adjusting their composition of certain transcripts, proteins, metabolites, and lipids (Mittler et al. 2012). Many proteins and heat shock proteins (Hsps) play key roles in the coping mechanisms of plants under abiotic stress. Of these adaptive mechanisms, the induction of antioxidant enzymes and osmoprotectants represents an important phenomenon that improves the tolerance of plants to HS.

NO, a small diffusible and ubiquitous molecule, improves the tolerance of plants to abiotic stress by activating a series of cellular signaling pathways. Furthermore, NO plays a pivotal role in several physiological processes, such as seed germination, secondary root initiation, plant height, stomatal closure, photosynthesis, and floral regulation, and also at the transcriptional levels (Siddiqui et al. 2011; Khan et al. 2012; Alavi et al. 2014; Dong et al. 2014). NO acts as an antioxidant (Karplus et al. 1991) and behaves like a hormone (Yamasaki et al. 2005). Under stress conditions, NO stimulates defensive mechanisms in plants in order to reduce oxidative damage by improving the antioxidant system and maintaining ROS balance (Simontacchi et al. 2015; Khan et al. 2017).

Among the auxins, IAA is a key phytohormone, which plays a vital role in plant growth and development processes, including cell division, elongation, cell differentiation, root initiation, apical dominance, and tropic responses (Sharma et al. 2015). Additionally, auxin plays an important role in regulating gene expression and ROS homeostasis under stress conditions (Joo et al. 2005; Sharma et al. 2015). Despite this, the physiological and molecular mechanisms of IAA action are still unknown. Pagnussat et al. (2004) reported that NO is involved in the auxin response during the adventitious rooting process in *Cucumis sativus* by activating mitogen-activated protein kinase (MAPK) cascades, which are universal signal transduction modules connecting extracellular stimuli to a wide range of cellular responses in plants. MAPKs participate in signaling in response to various biotic and abiotic stresses. In the present study, we show that NO acts as a signaling molecule in IAA-mediated tolerance of plants to HS through the activation of antioxidant enzymes, osmoprotectant (proline; Pro) synthesis, and enhanced accumulation of photosynthetic pigments. Additionally, co-treatment with NO and IAA suppresses DNA damage and ROS formation in tomato plants.

2. Materials and methods

2.1. Plant culture and SNP and/or IAA treatments

Tomato seeds (*L. esculentum* Mill. var. Five Star F-1 Hybrid) were obtained from the local market of Riyadh, Saudi Arabia. Before sowing, healthy seeds were surface-sterilized with 1% sodium hypochlorite for 10 min and then vigorously rinsed with sterilized double-distilled water (DDW). Tomato seeds were germinated on two sheets of sterilized filter paper in a 12-cm diameter Petri dish. The following treatments were applied to each Petri dish: (i) 0 µM SNP + 0 nM IAA, control; (ii) 100 µM SNP; (iii) 50 nM IAA; (iv) 100 µM SNP + 50 nM IAA; and (v) 100 µM SNP + 50 nM IAA + 200 µM cPTIO. SNP [$Na_2[Fe(CN)_5NO].2H_2O$] was used as a NO donor, while cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] was used as a NO scavenger. The concentrations of SNP (100 µM) and IAA (50 nM) were selected based on those used previously by Laspina et al. (2005) and Li et al. (2008), respectively. The Petri dishes were arranged in a sample-randomized design with a single factor and six replicates. All Petri dishes contained seven seedlings and were kept in a growth chamber (temperature 25 ± 3°C, relative humidity 50–60%, light 250 µmol photons m^{-2} s^{-1}, and a 16/8-h light/dark cycle). After 10 days of seed germination in Petri dishes, four healthy tomato seedlings were transferred to pots (6 cm diameter) containing sand/perlite (2:1), and grown under the same conditions. Finally, the pots were arranged in a sample-randomized design with a single factor and four replicates. The above treatments were applied to each pot with half-strength Hoagland's nutrient solution. After 7 days of transfer, tomato seedlings were subjected to HS by placing the pots in an incubator (38°C for 4 h).

2.2. Determination of the morphological characteristics of plants

Experimental plants were sampled after 20 days of HS treatments for morphological analysis. The growth performance of tomato plants was evaluated in terms of plant height (PH plant^{-1}), fresh and dry weight (FW and DW plant^{-1}), and area leaf $^{-1}$. Leaf area was measured directly using a Leaf Area Meter (LI-COR Inc., USA). The area of three leaves (upper, middle, and lower) from each plant of the sample (consisting of four plants) was determined.

2.3. Determination of the physio-biochemical characteristics of plants

Plants were sampled immediately after HS treatment for physio-biochemical analysis.

2.3.1. Chlorophyll

Chlorophyll (Chl) was extracted from fresh leaves of experimental plants using the dimethyl sulphoxide (DMSO) method based on that described by Barnes et al. (1992). Chl content in the extract was measured using a UV–vis spectrophotometer (SPEKOL 1500; Analytik Jena AG, Jena, Germany). The Chl content was calculated using the following formula:

$$\text{Chl } a = 14.85A_{664.9} - 5.14A_{648.2},$$

$$\text{Chl } b = 25.48A_{648.2} - 7.36A_{664.9}.$$

2.3.2. Proline

Pro concentration was estimated colorimetrically according to the method described by Bates et al. (1973) based on its reaction with ninhydrin. Fully expanded leaf samples were sampled and homogenized in 3% aqueous sulfosalicylic acid and the homogenate was then filtered. Two milliliters each of acid ninhydrin and glacial acetic acid were reacted with 2 mL of filtrate in a test tube for 1 h at 100°C. The mixture was extracted with toluene by mixing vigorously with a vortex and the free toluene content was quantified at 520 nm.

2.3.3. Malondialdehyde

Malondialdehyde (MDA) content was estimated according to the method described by Heath and Packer (1968). Leaf samples were weighed, and homogenates containing 10% trichloroacetic acid and 0.65% 2-thiobarbituric acid were heated at 95°C for 60 min, cooled to room temperature, and centrifuged at 10,000×*g* for 10 min. The absorbance of the supernatant was read at 532 and 600 nm against a reagent blank.

2.3.4. Electrolyte leakage

Electrolyte leakage (EL) was used to measure solute leakage (membrane permeability) in accordance with the methods described by Lutts et al. (1995). To remove surface contamination, leaf samples were washed three times with DDW. Leaf discs were cut from young leaves and placed in sealed vials containing 10 mL DDW, and then incubated on a rotary shaker for 24 h. The electrical conductivity of the solution (EC_1) was then determined. Next, the samples were autoclaved at 120°C for 20 min, and the electrical conductivity was measured again (EC_2) once the solution had cooled to room temperature. EL was defined as $EC_1/EC_2 \times 100$ and expressed as a percentage.

2.3.5. Determination of antioxidant enzyme activity

To measure the activity of antioxidant enzymes, leaf samples were homogenized in extraction buffer (0.5% Triton X-100 and 1% polyvinylpyrrolidone in 100 mM potassium phosphate buffer, pH 7.0) using a chilled mortar and pestle. The homogenates were then placed into individual centrifuge tubes and centrifuged at 15,000×*g* for 20 min at 4°C. The clear supernatant was used for enzymatic assays, which were performed at low temperature. Enzymatic activity was determined using a UV–vis spectrophotometer.

Peroxidase (POD) (EC 1.11.1.7) activity was determined according to the method described by Chance and Maehly (1955). Activity was assayed using 5 mL of the enzyme reaction solution containing phosphate buffer (pH 6.8), 50 M pyrogallol, 50 mM hydrogen peroxide (H_2O_2), and 1 mL of enzyme extract diluted 20X. The assay mixture was incubated for 5 min at 25°C. After incubation, the reaction was stopped by the addition of 0.5 mL 5% (v/v) H_2SO_4. The formation of purpurogallin was measured spectrophotometrically at 420 nm. One unit of POD activity was considered as the amount of purpurogallin formed per milligram of protein per minute.

The method described by Aebi (1984) was used to determined catalase (CAT) (EC 1.11.1.6) activity. Decomposition of H_2O_2 was measured as the decrease in absorbance at 240 nm. In this assay, 50 mM phosphate buffer (pH 7.8) and 10 mM H_2O_2 were used in the reaction solution.

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was measured based on the inhibition of nitroblue tetrazolium (NBT) according to the method described by Giannopolitis and Ries (1977). The reaction solution consisted of 50 mM NBT, 1.3 mM riboflavin, 13 mM methionine, 75 µM ethylenediamine tetraacetic acid (EDTA), 50 mM phosphate buffer (pH 7.8), and 20–50 mL enzyme extract. The reaction solution was irradiated under fluorescent light at 75 µM m^{-2} s^{-1} for 15 min. The absorbance of each reaction solution was read at 560 nm against a blank (non-irradiated reaction solution). One unit of SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photoreduction.

2.4. Histochemical detection of SNP and ROS in roots by fluorescence microscopy

NO was detected in the roots of treated plants using 4,5-diaminofluorescein diacetate (DAF-2DA) and fluorescence microscopy. Segments of primary roots (10 mm from the root tip) were incubated in a solution containing the fluorescent probe DAF-2DA (prepared in 10 m M Tris-HCl; pH 7.4) for 1 h in the dark at room temperature. Thereafter, roots were washed three times in fresh buffer without DAF-2DA and examined by excitation at 490 nm and emission at 525 nm under a microscope (Nikon Eclipse Ni-U, Nikon, Tokyo, Japan) (Rodríguez-Serrano et al. 2006).

H_2O_2 was imaged using the fluorescent probe 2,7′-dichlorofluorescein diacetate (DCF-DA) following the method described by Tarpey et al. (2004). H_2O_2 was detected by incubating roots with 25 µM DCF-DA (prepared in 10 mM Tris-HCl) for 30 min at 37°C. Thereafter, roots were washed three times with buffer and imaged using a fluorescence microscope at excitation and emission wavelengths of 480 and 530 nm, respectively.

2.5. Histochemical detection of ROS in leaves

O_2^- and H_2O_2 were detected in tomato leaves according to the methods described by Mostofa and Fujita (2013) and Wang et al. (2011). Briefly, the second leaves of treated plants were incubated in 0.1% NBT and 1% 3,3-diaminobenzidine (DAB) solutions (prepared in 50 mM tris acetate buffer; pH 5.8) for 12 h at room temperature in the dark and light, respectively. Thereafter, incubated leaves were decolorized by boiling in ethanol to detect blue insoluble formazan (for O_2^-) and the deep brown polymerization product (for H_2O_2). After cooling, the leaves were imaged by placing between two glass plates.

2.6. Determination of DNA damage in leaves under HS treatment

HS-induced DNA damage in tomato leaves was measured using the comet assay (single-cell gel electrophoresis), as described by Lin et al. (2007). Leaves from each treatment group were sampled, washed with DDW, and then used in the comet assay immediately after drying. This was performed under dim or yellow light to avoid light-induced DNA damage. Using a new razor blade, each leaf sample was gently cut into pieces in a Petri dish containing chilled phosphate-buffered saline (NaCl 130 mM, Na_2HPO_4 7 mM, NaH_2PO_4 3 mM, and EDTA 50 mM, pH 7.5). Nuclei present in the buffer were collected and used in the comet assay. The cell suspension was mixed with 75 µL of 0.5% low-melting agarose and layered on slides precoated with 1% normal melting agarose. Once the agarose had solidified, 75 µL of 0.5% low-melting agarose was layered. The prepared slides were immersed in a lysis solution containing a high salt concentration (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium laurylsarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4°C. Equilibration for 3×5 min in 1X Tris-Borate-EDTA (TBE) buffer on ice was followed by electrophoresis at room temperature in the same buffer for 6 min at 15–17 mA (Koppen et al. 1999). Each slide was stained with 50 µL of 13 mg L^{-1} SYBR green 1 and viewed using a fluorescent microscope (Eclipse Ni-U, Nikon, Tokyo, Japan)

Table 1. Effect of NO and IAA on the growth characteristics of tomato plants under stress and non-stress conditions.

	Parameters							
	Plant height (cm)	Leaf area (cm^2)	Fresh weight (g)	Dry weight (g)	Shoot length (cm)	Leaf area (cm^2)	Fresh weight (g)	Dry weight (g)
Treatments	Non-HS				HS			
Control	13.92 ± 0.34^{c}	06.87 ± 0.11^{d}	0.23 ± 0.005^{c}	0.014 ± 0.0010^{c}	09.50 ± 0.54^{c}	03.54 ± 0.29^{d}	0.11 ± 0.010^{c}	0.010 ± 0.0009^{c}
NO	17.75 ± 0.38^{b}	11.67 ± 0.33^{b}	0.31 ± 0.008^{b}	0.019 ± 0.0013^{ab}	14.75 ± 0.38^{b}	10.43 ± 0.41^{b}	0.24 ± 0.023^{b}	0.016 ± 0.0029^{b}
IAA	17.80 ± 0.38^{b}	10.52 ± 0.32^{c}	0.29 ± 0.009^{b}	0.018 ± 0.0010^{b}	13.53 ± 0.47^{b}	08.92 ± 0.43^{c}	0.23 ± 0.034^{b}	0.016 ± 0.0008^{b}
NO + IAA	21.10 ± 0.23^{a}	15.60 ± 0.31^{a}	0.42 ± 0.022^{a}	0.023 ± 0.0015^{c}	17.53 ± 0.39^{a}	14.52 ± 0.41^{a}	0.35 ± 0.021^{a}	0.022 ± 0.0015^{a}
cPTIO + NO + IAA	13.70 ±0.15^{c}	06.60 ± 0.19^{d}	0.22 ± 0.009^{c}	0.016 ± 0.0012	08.28 ± 0.66^{c}	03.23 ± 0.15^{d}	0.10 ± 0.011^{c}	0.008 ± 0.0006^{c}

Notes: Data followed by the same letters are not significantly different at $p < .05$%. Average of four determinations is presented with data showing SE.

with an excitation/emission filter of 496/522 nm. Comet images were captured by a camera (DS-Ri1). Comets were examined using the image software ImageJ (http://rsbweb.nih.gov/ij/). The level of DNA damage was expressed as % DNA in tail and tail movement.

The data were expressed as the mean ± standard error and were analyzed statistically using SPSS Ver. 17 statistical software. The means were compared statistically using Duncan's multiple range test at the level of $p < .05$.

3. Results

The effect of co-treatment with SNP and IAA was assessed on the basis of morphological, physiological, and biochemical characteristics of the tomato plant under HS and non-HS conditions.

3.1. Morphological characteristics

Four growth characteristics of plants were measured (PH plant^{-1}, FW and DW plant^{-1}, and area leaf $^{-1}$; Table 1, Figure 1). The application of SNP and IAA alone, as well as in combination, enhanced all growth characteristics under the non-stress condition. Moreover, all four growth parameters were reduced as compared to the control in response to HS. Under non-HS conditions, the co-application of SNP and IAA enhanced PH plant^{-1}, FW and DW plant^{-1}, and area leaf^{-1} by 51.58%, 127.10%, 82.61%, and 64.28%, respectively, compared with their respective controls. Additionally, exogenous application of SNP + IAA increased PH plant^{-1}, FW and DW plant^{-1}, and area leaf^{-1} by 84.53%, 310.17%, 218.18%, and 120%, respectively, compared with their respective controls under HS conditions. Under both conditions (stress and non-stress), the co-application of SNP and IAA was more effective in enhancing the growth characteristics of plants. In order to validate the beneficial effect of SNP, the SNP scavenger cPTIO was added with SNP. We found that the enhancing effect of SNP on the growth parameters was reversed (Table 1) under both conditions.

3.2. Physiological and biochemical characteristics

The synthesis of photosynthetic pigments (Chl *a* and *b*) and Pro was increased in plants treated with SNP and IAA, alone as well as in combination (Table 2), under non-stress conditions. HS significantly inhibited the levels of Chl *a* and *b* in leaves as compared to their respective controls. However, plants treated with SNP and/or IAA showed enhanced values for Chl *a*, *b* and Pro under HS. Under stress and non-stress conditions, the co-application of SNP and IAA enhanced Chl *a* content by 93.32% and 40.29%, Chl *b* by 132.25% and 82.44%, and Pro 152% and 111.21%, respectively, compared with their respective controls. In the presence of cPTIO, the SNP-treated plants showed reduced levels of both photosynthetic pigments and Pro under both conditions.

Figure 1. Effect of NO and IAA on the growth of tomato plants under HS.

Table 2. Effect of NO and IAA on the accumulation of Chl *a* and *b*, and Pro of tomato plants under stress and non-stress conditions.

	Parameters					
	Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	Proline (µg^{-1} FW)	Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	Proline (µg^{-1} FW)
Treatments	Non-HS			HS		
Control	30.83 ± 1.10^{c}	06.32 ± 0.39^{c}	1.16 ± 0.107^{c}	20.67 ± 1.41^{c}	04.31 ± 0.34^{d}	1.50 ± 0.24^{c}
NO	42.38 ± 0.70^{ab}	09.35 ± 0.51^{b}	1.85 ± 0.181^{b}	29.87 ± 0.94^{b}	08.61 ± 0.26^{b}	2.98 ± 0.07^{b}
IAA	39.23 ± 0.78^{b}	09.46 ± 0.43^{b}	1.70 ± 0.153^{b}	32.74 ± 1.45^{b}	07.23 ± 0.56^{c}	2.70 ± 0.15^{b}
NO + IAA	43.25 ± 1.60^{a}	11.53 ± 0.70^{a}	2.45 ± 0.254^{a}	39.96 ± 0.77^{a}	10.01 ± 0.46^{a}	3.78 ± 0.17^{a}
cPTIO + NO + IAA	30.12 ± 0.80^{c}	05.75 ± 0.37^{c}	1.04 ± 0.086^{c}	20.44 ± 1.02^{c}	04.15 ± 0.28^{d}	1.72 ± 0.31^{c}

Notes: Data followed by the same letters are not significantly different at $p < .05\%$. Average of four determinations are presented with data showing SE.

Figure 2(A–C) reveals that heat-stressed plants exhibited more DNA damage than non-heat-stressed plants. However, the co-application of SNP and IAA was found to be effective in alleviating the adverse effects of HS by protecting against DNA damage. The application of cPTIO with SNP enhanced the DNA damage signal, and confirmed the involvement of SNP in the tolerance of plants to HS by reducing DNA damage. The comet traits (tail movement and tail DNA%) significantly increased under HS. However, the co-application of SNP and IAA decreased these comet parameters. In the present study, the results confirm that the inclusion of the SNP scavenger cPTIO in solution eliminated the ameliorating effect of SNP on comet characteristics under HS conditions.

In situ production of ROS in plant leaves was visualized using DAB staining following HS exposure (Figure 3). We observed that DAB polymerized and generated a deep brown color in leaves in the presence of H_2O_2. The production of DAB-H_2O_2 in intact leaves of HS-treated plants was higher than that in the leaves of non-HS-treated plants. However, the DAB-H_2O_2 reaction product developed to a lesser extent in the leaves of plants treated with SNP + IAA as compared to the control and cPTIO-treated plants. Additionally, a similar result was recorded for the formation of O_2^- in the leaves of tomato plants treated with SNP and/or IAA (Figure 4). The production of O_2^- in leaves was visualized using NBT as the formation of a dark-blue insoluble formazan. We observed that there was less O_2^- production in the leaves of non-HS-treated plants compared with those of HS-treated plants. However, the leaves of SNP + IAA-treated plants were stained less blue as compared to those subject to other treatments under HS. In the present study, we used DCF-DA to visualize ROS (H_2O_2) formation in the roots of treated plants under both conditions (Figure 5(A)). After 4-h HS, a sharp increase in the green DCF fluorescent signal was observed in roots as compared to those from non-HS-treated plants. However, some green DCF fluorescence was observed in the roots of plants treated with SNP and/or IAA. Conversely, cPTIO was added to the medium to confirm the protective role of SNP against ROS formation in plants subjected to HS. We observed that the pattern of green fluorescence reversed and was similar to that of the control. Therefore, we postulated that SNP may be effective at mitigating the effects of ROS.

The observed MDA content in leaves and EL indicated the occurrence of lipid peroxidation and membrane damage in

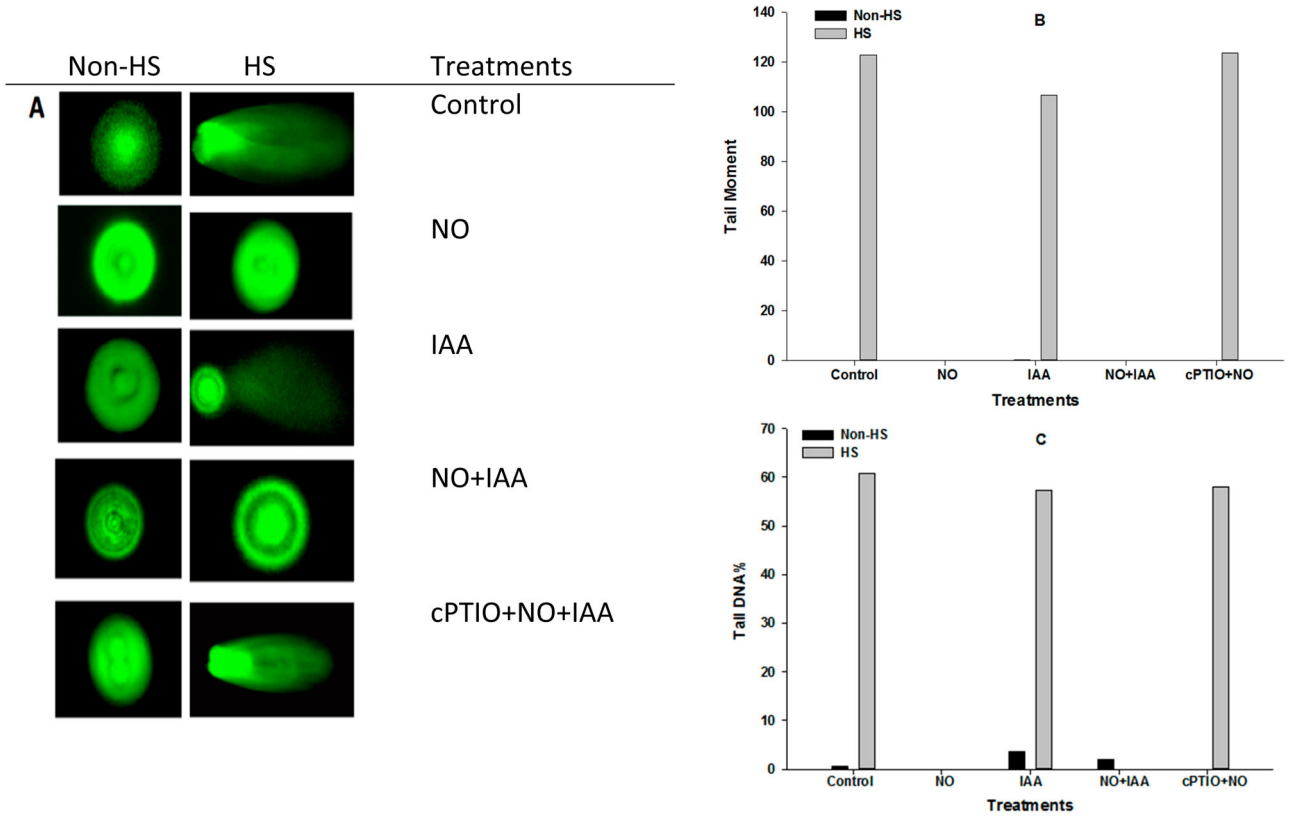

Figure 2. HS-induced DNA damage (A) in the leaves of tomato plants detected by the comet assay at the single-cell level under a neutral conditions protocol. Under HS, NO and/or IAA, and cPTIO (NO scavenger) induced changes in DNA damage ((B) tail DNA% and (C) tail movement) in the leaves of tomato plants.

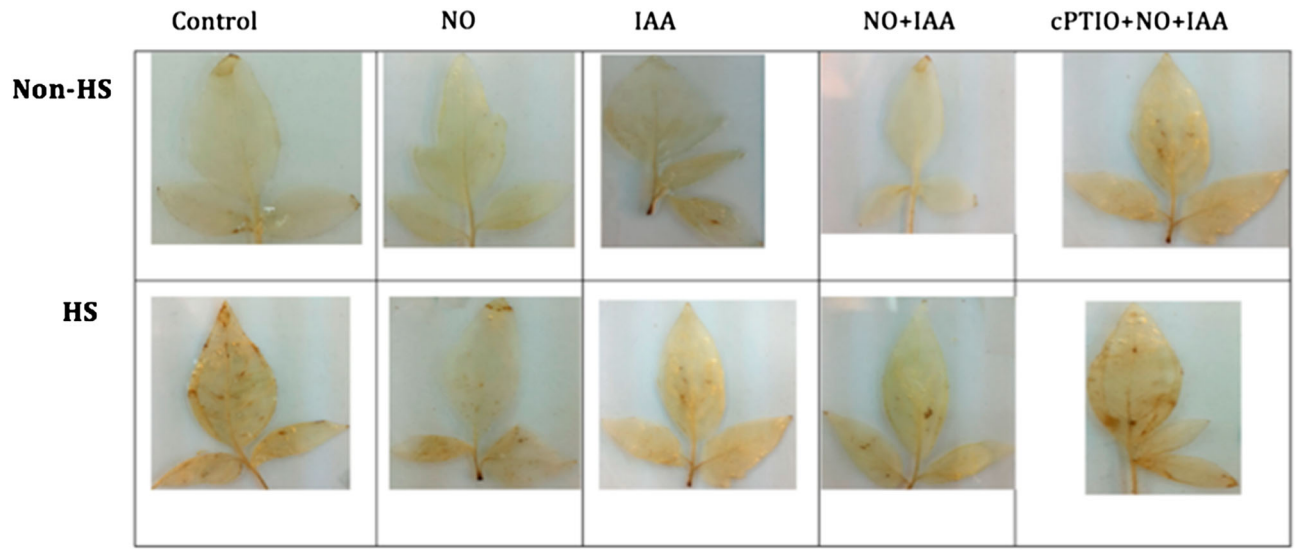

Figure 3. *In situ* detection of H_2O_2 using DAB staining under HS and non-HS conditions. Brown color showing H_2O_2 formation in the leaves of tomato plants.

experimental plants (Figure 5(C,D)). In general, HS conditions had a more detrimental effect on plants than non-HS conditions. However, under HS, the application of SNP and IAA, alone as well as in combination, inhibited the levels of MDA and EL. The accumulation of MDA and EL were decreased by 47.33% and 40.84%, respectively, compared with their respective controls under HS conditions. In the present study, we confirmed that the inclusion of cPTIO in the medium eliminated the ameliorating effect of SNP under HS conditions.

In order to evaluate the ameliorating role of SNP in plant growth and development under non-HS and HS conditions, the production of SNP in root tips was visualized using DAF-2DA fluorescence intensities (Figure 5(B)). Under HS, the roots of control plants exhibited low levels of signal molecules (indicated by DAF fluorescence intensities) compared with those from plants treated with SNP and IAA alone as well as in combination. The level of DAF-2DA fluorescence intensity was higher in roots from plants treated with the combination of SNP and IAA compared with that observed when treated alone under both conditions. Moreover, following the addition of the SNP scavenger cPTIO to SNP, the root displayed a similar DAF fluorescence intensity to the control. Thus, we speculate that SNP could be involved in mediating a positive effect on plants by mitigating oxidative damage induced by HS. In the present study, it is interesting that the DAF fluorescence intensity was higher in the root of plants co-treated with SNP and IAA than in those of plants treated with SNP and IAA alone.

The effect of SNP and/or IAA on antioxidant enzyme activity (CAT, POD, and SOD) in tomato plants was analyzed. Figure 6 shows there was a marked increase in the activity of these enzymes in plants treated with SNP and/or IAA under non-HS conditions. However, the co-application of SNP and IAA enhanced these activities further under HS conditions. The co-application of SNP and IAA enhanced the activity of CAT, POD, and SOD by 35.62%, 25.42%, and 121.17% under non-HS conditions, and by 31.61%, 26.42%, and 80.03% under HS conditions, respectively, compared with their respective controls. Conversely, cPTIO in combination with SNP significantly nullified the enhancing effect of SNP, resulting in a decrease in the activity of antioxidant enzymes.

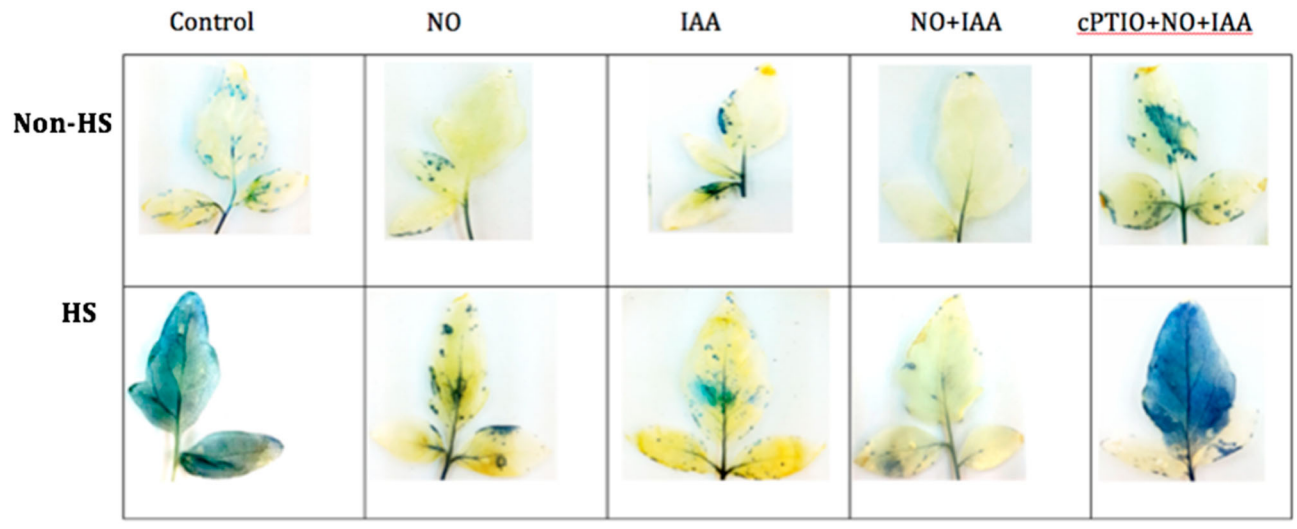

Figure 4. *In situ* visualization of O_2^- generation in leaves using NBT staining under HS and non-HS conditions. Blue color indicating O_2^- formation in the leaves of tomato plants.

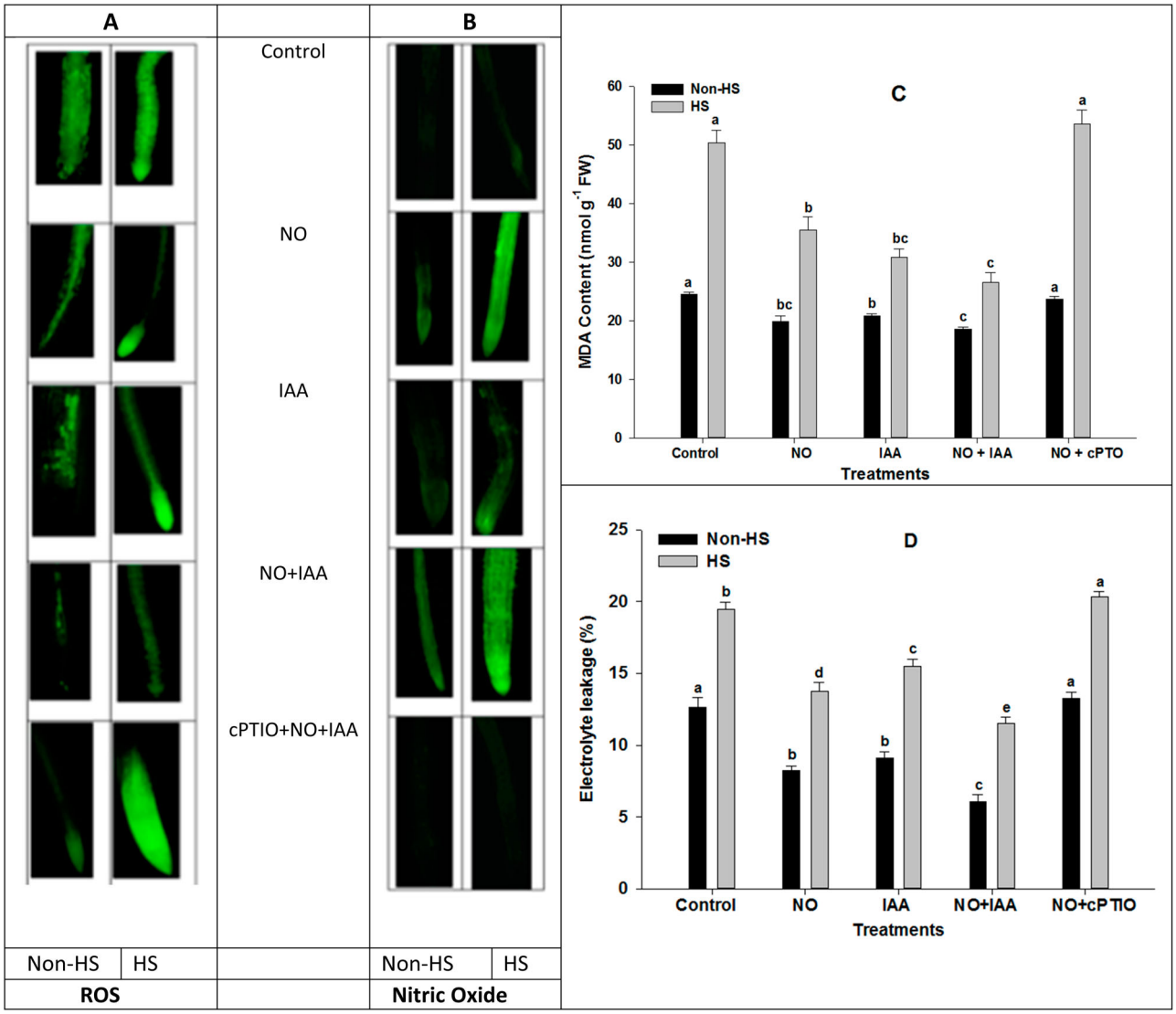

Figure 5. *In situ* visualization of (A) ROS and (B) NO in roots under HS and non-HS conditions. Effect of NO and IAA on the (C) content of MDA and (D) EL of tomato plants under stress and non-stress conditions. Bars followed by the same letter do not differ statistically at $p < .05$ (Duncan's multiple range test). Average of four determinations is presented, with bars indicating SE.

4. Discussion

The application of SNP and IAA, alone as well as in combination, to tomato seedlings enhanced growth (Figure 1, Table 1) and physiological and biochemical parameters (Table 2, Figure 6) under non-HS conditions. Plants performed poorly under HS; however, the combined application of both SNP and IAA restored the physio-biochemical traits of tomato seedlings.

In this study, HS decreased plant growth parameters, which may be explained by the formation of ROS, by changes in photosynthesis, respiration, water relations, and membrane stability, and also by disrupted hormonal balance, primary and secondary metabolites, and altered anatomical structure in plants (Wahid et al. 2007). However, under both non-HS and HS conditions, we found that the application of SNP and IAA individually, as well as in combination, to tomato plants ameliorated these growth characteristics (Table 1, Figure 1). The growth-enhancing effect of SNP and/or IAA may be explained by their physiological roles. It is well known that NO is required for optimal root growth and regulates the transduction of the auxin signaling pathway (Correa-Aragunde et al. 2007), and both are required for cell elongation, cell division, and tissue differentiation (Fernández-Marcos et al. 2012), which lead to optimal plant growth. The application of SNP and IAA together was found to be more effective than their application alone; this may be due to the fact that SNP suppresses auxin degradation by inhibiting the IAA degrading enzyme (IAA oxidase) and also regulates auxin-mediated processes during plant growth (Fernández-Marcos et al. 2012; Simontacchi et al. 2013). In the present experiment, enhanced growth characteristics were inhibited by the application of the specific SNP scavenger (cPTIO) with SNP. These findings confirm that SNP plays a significant role in the regulation of auxin-induced plant growth (Figure 1). Pagnussat et al. (2004) reported that exogenous application of SNP improved IAA-mediated root growth through the activation of cGMP and MAPK signaling in plants. Thus, we can postulate that the application of SNP with IAA was more effective at reversing the altered plant growth induced by HS.

In the present experiment, we explored the combined effect of SNP and IAA on the accumulation of photosynthetic pigments (Chl *a* and *b*) and an osmolyte (Pro) under non-HS and HS conditions (Table 2). Significant changes in the accumulation of both chlorophylls and Pro were observed

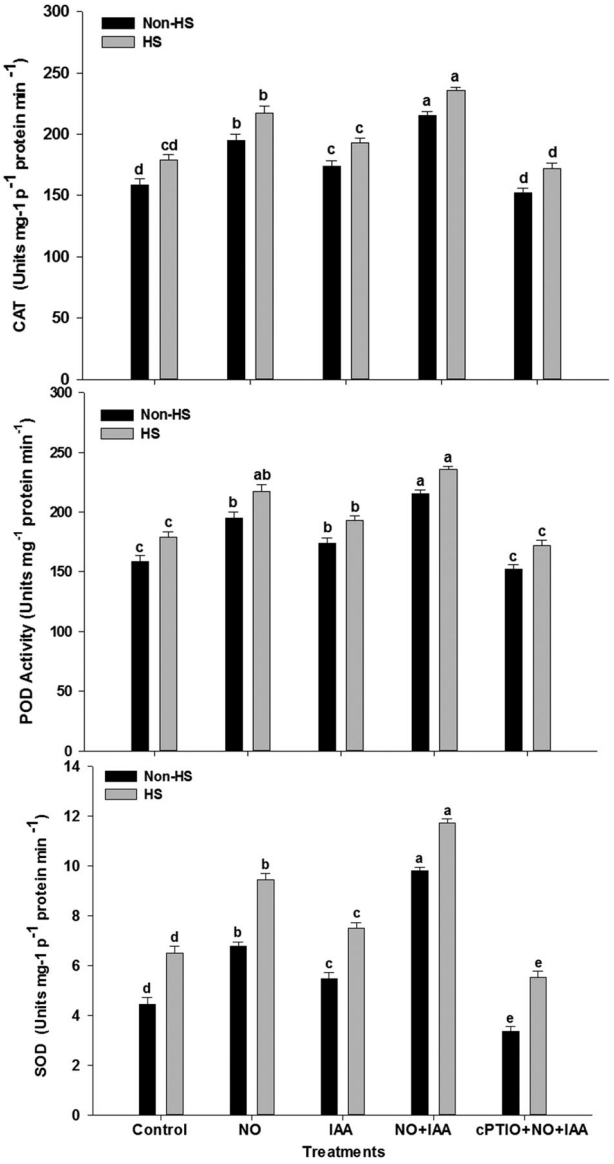

Figure 6. Effect of NO and IAA on the activity of antioxidant enzymes under stress and non-stress conditions. Bars followed by the same letter do not differ statistically at $p < .05$ (Duncan's multiple range test). Average of four determinations is presented, with bars indicating SE.

following the application of SNP and/or IAA under both conditions. The synthesis of both Chl *a* and *b* pigments and Pro was enhanced by the application of SNP and/or IAA under non-HS conditions; however, Chl *a* and *b* contents were severely affected under HS conditions. Decreased Chl *a* and *b* contents were probably due to impaired 5-aminolevulinic acid biosynthesis and the destruction of chloroplasts (Gosavi et al. 2014). However, interestingly, we found that the application of SNP and IAA individually as well as in combination notably improved the synthesis of photosynthetic pigments, and may thus improve the tolerance of plants to HS. These results substantiate the findings of Khan et al. (2012) and Siddiqui et al. (2013). NO plays a vital role in maintaining iron homeostasis and accelerates internal iron transport, resulting in the synthesis of photosynthetic pigments and chloroplast development (Graziano & Lamattina 2005). We found that the inclusion of IAA with SNP may have enhanced the contents of Chl *a* and *b*, and Pro more than when either was added alone. SNP protects IAA from degradation and regulates auxin-dependent gene expression through interaction with the TIR1/AFBs auxin receptor (TAAR) proteins, which could improve the tolerance of plants to HS by increasing the chlorophyll content, germination, and root development (Iglesias et al. 2014). Kabir et al. (2013) reported that chlorophyll synthesis was blocked when an auxin inhibitor (2,3,5-triiodobenzoic acid) was administered with auxin treatment. To confirm the role of SNP in association with IAA, SNP plus NO scavenger (cPTIO) was added to plants and similar results were obtained for both chlorophylls and Pro accumulation to that of the control under non-HS and HS conditions. Under stress conditions, the hyperaccumulation of Pro in plants following the application of SNP and/or IAA led to enhanced resistance to HS through the maintenance of an energy source and regulation of gene expression for osmotic adjustment (Khan et al. 2012).

DNA damage can be considered a biomarker of genotoxicity in the leaves of tomato plants exposed to HS. Interestingly, we found that plants subjected to HS had significantly more DNA strand breakages than control plants (Figure 2(A,B)). This may be due to direct or indirect HS-induced DNA damage in leaves through the production of ROS (Figures 3, 4, and 5(A)), which react with cell components resulting in cell death (Potters et al. 2010). This result corroborates a recent finding by Cvjetko et al. (2014) who used the comet assay and observed HS-induced DNA damage in the leaves of tobacco plants. In the present study, the level of DNA damage was correlated with the formation of ROS (Figures 3, 4, and 5(A,C)). However, the application of SNP and/or IAA significantly reduced the degree of DNA damage. This may be due to the accumulation of NO in the root and enhanced activities of antioxidant enzymes (Figures 5(B), 6). An increase in the activities of antioxidant enzymes may have been responsible for reduced levels of DNA damage by preventing the reaction of ROS with the leaf nuclear DNA (Gichner et al. 2008). Conversely, the inclusion of cPTIO with SNP and IAA confirms the cytoprotective role of SNP in reducing the DNA damage induced by HS.

Under different environmental stress conditions, oxidative damage has been measured by estimating the contents of MDA, H_2O_2, and O_2^-, and EL. In the present experiment, the increased values found for MDA, EL, and the formation of H_2O_2 and O_2^- in the roots and leaves of plants under HS conditions were indicative of HS-induced cellular dysfunction through the induction of lipid peroxidation (Figures 3, 4, 5(A)). An increase in MDA, H_2O_2, O_2^-, and EL due to HS was also observed by Siddiqui et al. (2015) in *Vicia faba*. Overproduction of ROS under stress conditions causes cellular damage and invokes the Haber–Weiss reaction, resulting in the formation of hydroxyl radicals and thus lipid and pigment peroxidation, which compromise membrane permeability and function (Mittler et al. 2012; Siddiqui et al. 2015). However, the application of SNP and IAA alone, as well as in combination, might be effective in reducing the impact of these factors by enhancing the activities of antioxidant enzymes such as POD, CAT, and SOD in plants (Figure 6). In the present study, the activity of POD, CAT, and SOD was highest in plants receiving SNP + IAA (Figure 6). The antioxidant system plays a significant role in maintaining steady-state levels of ROS in plants. The application of SNP may act as an antioxidant and suppress oxidative damage by reacting with oxygen species, thiols, hemes, and proteins to generate biochemical signals that directly and indirectly regulate enzymatic activity (Siddiqui et al. 2011). The addition of the NO scavenger cPTIO to plants completely

abrogated the effect of SNP on the accumulation of MDA, H_2O_2, O_2^-, and antioxidant enzymes. These results confirm the role of NO in plant growth and development by maintaining the ratio of cellular anti- and pro-oxidants.

5. Conclusion

In conclusion, the results of this study showed that the application of SNP had a substantial synergistic response on plant growth when supplied in conjunction with IAA. This could be explained by enhanced growth characteristics, because the co-application of SNP and IAA proved more effective than single application in enhancing the biosynthesis of photosynthetic pigments and Pro under both non-stress and HS conditions. The co-application of SNP and IAA enhanced the activity of antioxidant enzymes and the generation of NO in tomato seedlings, resulting in the prevention of ROS and DNA damage in tomato plants, thus improving the tolerance of the plants to HS. However, the addition of the SNP scavenger cPTIO confirms a protective role of SNP in association with IAA.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Aebi, H. 1984. Catalase *in vitro*. Methods Enzymol. 105:121–126.

Alavi SMN, Arvin MJ, Kalantari KM . 2014. Salicylic acid and nitric oxide alleviate osmotic stress in wheat (*Triticum aestivum* L.) seedlings. J Plant Interact. 9:683–688.

Barnes JD, Balaguer L, Manrique E, Elvira S, Davison AW . 1992. A reappraisal of the use of DMSO for the extraction and determination of chlorophylls a and b in lichens and higher plants. Environ Exp Bot. 32:85–100.

Bates LS, Waldren RP, Teare ID . 1973. Rapid determination of free proline for water-stress studies. Plant Soil. 39:205–207.

Chance B, Maehly AC . 1955. Assay of catalase and peroxidases. Methods Enzymol. 2:764–775.

Correa-Aragunde N, Lanteri ML, GarcÍa-Mata C, TenHave A, Laxalt AM, Graziano M, Lamattina L . 2007. Nitric oxide functions as intermediate in auxin, abscisic acid, and lipid signaling pathways. In: Lamattina L, Polacco JC , editors. Plant cell monographs, nitric oxide in plant growth, Heidelberg. Berlin: Springer-Verlag; p. 113–130.

Cvjetko P, Balen B, Štefanić PP, Debogović L, Pavlica M, Klobučar GIV . 2014. Dynamics of heat-shock induced DNA damage and repair in senescent tobacco plants. Biol Plant. 58:71–79.

Dong Y, Xu L, Wang Q, Fan Z, Kong J, Bai X . 2014. Effects of exogenous nitric oxide on photosynthesis, antioxidative ability, and mineral element contents of perennial ryegrass under copper stress. J Plant Interact. 9:402–411.

Fernández-Marcos M, Sanz L, Lorenzo Ó . 2012. Nitric oxide: an emerging regulator of cell elongation during primary root growth. Plant Signal Behav. 7:196–200.

Garmin H . 2014. Tomatoes are the superlative vegetable: global per capita consumption is 20 kilograms per year, (2014, March 31). Available from: http://www.agribenchmark.org/agri-benchmark/did-youknow/einzelansicht/artikel//tomatoes-are.html

Giannopolitis CN, Ries SK . 1977. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59:309–314.

Gichner T, Patková Z, Száková J, Žnidarc I, Mukherjee A . 2008. DNA damage in potato plants induced by cadmium, ethyl methanesulphonate and γ-rays. Environ Exp Bot. 62:113–119.

Gosavi GU, Jadhav AS, Kale AA, Gadakh SR, Pawar BD, Chimote VP . 2014. Effect of heat stress on proline, chlorophyll content, heat shock proteins and antioxidant enzyme activity in sorghum (*Sorghum bicolor*) at seedlings stage. Indian J Biotechnol. 3:356–363.

Grass L, Burris JS . 1995. Effect of heat stress during seed development and maturation on wheat (*Triticum durum*) seed quality, I. Seed germination and seedling vigor. Can J Plant Sci. 75:821–829.

Graziano M, Lamattina L . 2005. Nitric oxide and iron in plants: an emerging and converging story. Trends Plant Sci. 10:4–8.

Heath RL, Packer L . 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 125:189–198.

Hemantaranjan A, Bhanu AN, Singh MN, Yadav DK, Patel PK, Singh R, Katiyar D . 2014. Heat stress responses and thermotolerance. Adv Plants Agri Res. 3:1–10.

Iglesias MJ, Terrile MC, Windels D, Lombardo MC, Bartoli CG, Vazquez F, Estelle M, Casalongué CA, Rahman A . 2014. MiR393 regulation of auxin signaling and redox-related components during acclimation to salinity in Arabidopsis. PLoS One. 9:e107678. doi:10.1371/journal.pone.0107678.

Joo JH, Yoo HJ, Hwang I, Lee JS, Nam KH, Bae YS . 2005. Auxin-induced reactive oxygen species production requires the activation of phosphatidylinositol 3-kinase. FEBS Lett. 579:1243–1248.

Kabir AH, Paltridge NG, Rossener U, Stangoulis JCR . 2013. Mechanisms associated with Fe-deficiency tolerance and signalling in shoots of *Pisum sativum* L. Physiol Plant. 147:381–395.

Karplus PA, Daniels MJ, Herriot JR . 1991. Atomic structure of ferredoxin-NADP+ reductase, prototype for a structurally novel flavoenzyme family. Science. 251:60–66.

Khan MN, Mobin M, Abbas ZK, Siddiqui MH . 2017. Nitric oxide-induced synthesis of hydrogen sulfide alleviates osmotic stress in wheat seedlings through sustaining antioxidant enzymes, osmolyte accumulation and cysteine homeostasis. Nitric Oxide. doi.org/10.1016/j.niox.2017.01.001.

Khan M, Siddiqui MH, Mohammad F, Naeem M . 2012. Interactive role of nitric oxide and calcium chloride in enhancing tolerance to salt stress. Nitric Oxide. 27:210–218.

Koppen G, Toncelli LM, Triest L, Verschaeve L . 1999. The comet assay: a tool to study alteration of DNA integrity in developing plant leaves. Mech Ageing Dev. 110:13–24.

Laspina NV, Groppa MD, Tomaro ML, Benavides MP . 2005. Nitric oxide protects sunflower leaves against Cd-induced oxidative stress. Plant Sci. 169:323–330.

Lavania D, Dhingra A, Siddiqui MH, Al-Whaibi MH, Grover A . 2015. Current status of the production of high temperature tolerant transgenic crops for cultivation in warmer climates. Plant Physiol Biochem. 86:100–108.

Li L, Hou X, Tsuge T, Ding M, Aoyama T, Oka A, Gu H, Zhao Y, Qu LJ . 2008. The possible action mechanisms of indole-3-acetic acid methyl ester in *Arabidopsis*. Plant Cell Rep. 27:575–584.

Lin AJ, Zhang XH, Chen MM, Qing CAO . 2007. Oxidative stress and DNA damages induced by cadmium accumulation. J Environ Sci. 19:596–602.

Lutts S, Kinet JM, Bouharmont J . 1995. Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. J Exp Bot. 46:1843–1852.

Mittler R, Finka A, Goloubinoff P . 2012. How do plants feel the heat? Trends Biochem Sci. 37:118–125.

Moore EL, Thomas WO . 1952. Some effects of shading and para-chlorophenoxy acetic acid on fruitfulness of tomatoes. P Am Soc Hort Sci. 60:289–294.

Mostofa MG, Fujita M . 2013. Salicylic acid alleviates copper toxicity in rice (*Oryza sativa* L.) seedlings by up-regulating antioxidative and glyoxalase systems. Ecotoxicology. 22:959–973.

Pagnussat GC, Lanteri ML, Lombardo MC, Lamattina L . 2004. Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. Plant Physiol. 135:279–286.

Potters G, Horemans N, Jansen MAK . 2010. The cellular redox state in plant stress biology – a charging concept. Plant Physiol Biochem. 48:292–300.

Rivero RM, Ruiz JM, Garcı´a PC, López-Lefebre LR, Sánchez E, Romero L . 2001. Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. Plant Sci. 160:315–321.

Rodríguez-Serrano María, Romero-Puertas MC, Zabalza ANA, Corpas FJ, Gómez M, Del Rio LA, Sandalio LM . 2006. Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. Plant Cell Environ. 29:1532–1544.

Sánchez B, Rasmussen A, Porter JR . 2014. Temperatures and the growth and development of maize and rice: a review. Global Change Biol. 20:408–417.

Sangu E, Tibazarwa FI, Nyomora A, Symonds RC . 2015. Expression of genes for the biosynthesis of compatible solutes during pollen development under heat stress in tomato (*Solanum lycopersicum*). J Plant Physiol. 178:10–16.

Sharma E, Sharma R, Borah P, Jain M, Khurana JP . 2015. Emerging roles of auxin in abiotic stress responses. In: GK Pandey, editor. Elucidation of abiotic stress signaling in plants. New York: Springer; p. 299–328.

Siddiqui MH, Al-Khaishany MY, Al-Qutami MA, Al-Whaibi MH, Grover A, Ali HM, Al-Wahibi MS . 2015. Morphological and physiological characterization of different genotypes of faba bean under heat stress. Saudi J Biol Sci. 22:656–663.

Siddiqui MH, Al-Whaibi MH, Ali HM, Sakran AM, Basalah MO, Al Khaishany MYY . 2013. Mitigation of nickel stress by the exogenous application of salicylic acid and nitric oxide in wheat. Aust J Crop Sci. 7:1780–1788.

Siddiqui MH, Al-Whaibi MH, Basalah MO . 2011. Role of nitric oxide in tolerance of plants to abiotic stress. Protoplasma. 248:447–455.

Simontacchi M, Galatro A, Ramos-Artuso F, Santa-María GE . 2015. Plant survival in a changing environment: the role of nitric oxide in plant responses to abiotic stress. Front Plant Sci. 6:4368. doi.org/10.3389/fpls.2015.00977.

Simontacchi M, García-Mata C, Bartoli CG, Santa-María GE, Lamattina L . 2013. Nitric oxide as a key component in hormone-regulated processes. Plant Cell Rep. 32:853–866.

Suzuki N, Bassil E, Hamilton JS, Inupakutika MA, Zandalinas SI, Tripathy D, Luo Y, Dion E, Fukui G, Kumazaki A, et al. 2016. ABA is required for plant acclimation to a combination of salt and heat stress. PLoS One. 1–21. doi:10.1371/journal.pone.0147625.

Tarpey MM, Wink DA, Grisham MB . 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: *in vitro* and *in vivo* considerations. Am J Physiol Regul Integr Comp Physiol. 286:431R–444.

Teixeira EI, Fischer G, van Velthuizen H, Walter C, Ewert F . 2013. Global hot-spots of heat stress on agricultural crops due to climate change. Agric Forest Meteorol. 170:206–215.

Wahid A, Gelani S, Ashraf M, Foolad MR . 2007. Heat tolerance in plants: an overview. Environ Exp Bot. 61:199–223.

Wang C, Zhang S, Wang P, Hou J, Qian J, Ao Y, Lu J, Li L . 2011. Salicylic acid involved in the regulation of nutrient elements uptake and oxidative stress in *Vallisneria natans* (Lour.) *Hara* under Pb stress. Chemosph. 84:136–142.

Yamasaki S, Fujii N, Takahashi H. 2005. Hormonal regulation of sex expression in plants. Vitam Horm. 72:79–110.