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Contribution of Glutathione and Ascorbate to Realization of the Protective Effect of Nitric Oxide on Wheat Plants under Drought †

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Abstract: Nitric oxide (NO) is an important signaling molecule capable of increasing the plant resistance to environmental stress of different nature. However, the mechanisms underlying the protective effect of NO on the plants still remains poorly understood. In the present study we have investigated the effect of 200 μmol SNP (Sodium Nitroprusside) on the growth and ascorbate acid (AsA) and glutathione (GSH) metabolism in wheat plants (*Triticum aestivum* L.) under the influence of 12 % PEG. The results showed that application of SNP to wheat plants increased AsA and GSH contents and enhancement of glutathione reductase activities. Meanwhile, growth stabilization and a decrease in the damaging effect of drought on the state of membrane structures were noted in plants, as judged by the level of MDA in them. Our results suggested that SNP can regulate the ascorbate and glutathione metabolism and has important roles in alleviating oxidative damage and enhancing drought tolerance in wheat.

Keywords: *Triticum aestivum* L.; ascorbate acid; glutathione; drought; nitric oxide

1. Introduction

Moisture deficiency or drought is one of the most common and critical environmental factors for plants, causing a sharp decline in the quality and productivity of many crops, including wheat, which is an important grain crop in the world. The use of various growth regulators in order to increase the stability and yield of wheat is an urgent task. One of the candidates for the role of a plant growth regulator with pronounced anti-stress effects is the nitric oxide (NO) molecule, in which the interest of scientists as plant physiologists and biochemists has not weakened for more than two decades. NO is an intracellular signaling molecule involved in the regulation of basic physiological processes at all stages of the plant life cycle, participating in the regulation of seed germination, root formation, gravitropism, stomata closure, flowering, fruit ripening, aging processes [1]. There is information about the ability of NO to increase plant resistance to a wide range of stress effects of biotic and abiotic nature [2,3]. The protective effect of NO donors can be the basis for the creation of chemical and biological preparations that increase the resistance of plants to moisture deficit and other abiotic stressors. Studies of the protective effect of nitric oxide on plant objects are relevant to this day, since, despite the large amount of literature data on the protective role of nitric oxide and the mechanisms of NO action as a signaling molecule in plant organisms, still unclear. In the course of previous studies, we selected the concentration of the donor of nitric oxide—Sodium Nitroprusside (SNP)—200 μM, which had growth-stimulating and anti-stress effects on wheat plants, which were judged by the growth parameters and the balance of phytohormones in the seedlings [4]. The aim of this work was to investigate the contribution of non-enzymatic antioxidants of the cell to the

formation of resistance in pretreated and untreated 200 μM SNP plants of wheat under the action of 12% PEG, in this regard, the analysis of the content of ascorbate acid (AsA), glutathione (GSH), malondialdehyde (MDA) and activity glutathione reductase (GR) in these seedlings.

2. Experiments

2.1. Plant Materials

The object of the study was wheat seedlings of Triticum aestivum L. cultivar Salavat Yulaev. The seeds were sterilized with 96% ethanol and germinated on filter paper moistened with tap water at 21–23 °C, 16-h photoperiod and illumination of 320 μmol/(m^2·s) FAR. 3-day-old seedlings were isolated from the endosperm; some of the seedlings were incubated for 24 h in a solution of 1.5% sucrose containing 200 μM SNP in the mixture, the other part only on 1.5% sucrose. Then, SNP-treated and untreated 4-day-old seedlings were exposed to 12% polyethylene glycol (PEG), which simulates moisture deficitconditions. Seedlings incubated in distilled water served as control in all variants of experiments.

2.1.2. Glutathione Determination

The content of reduced (GSH) form of glutathione from plant was determined using the spectrofluoromeric method based on obtaining a fluorescent product of O-phthalaldehyde (OPT) (Sigma, Australia) were recorded at pH 8.0 in the medium of measuring [5]. 0.5 g fresh roots were ground with 4 mL of a mixture consisting of 0.1 M potassium phosphate buffer (pH 8.0) and a 25% metaphosphoric acid solution in a ratio of 3.75:1 (by volume) as recommended by Hissin and Hilf [1976], then centrifuged at 12,000× *g* for 20 min at 4 °C. For the measurement of GSH, 0.5 mL of the supernatant was mixed with 4.5 mL of 0.1 M phosphate buffer (pH 8.0, including 5 mM EDTA), then take about 0.1 mL from this mixture and 1.8 mL of 0.1 M phosphate buffer (pH 8.0) and 0.1 mL of OPT stock (1 mg mL^{-1} in ethanol) added, fluorescencewas read at an excitation and emission wavelength of 350 and 420 nm using a Perkin Elmer LS 55 Luminescence Spectrometr Cell (USA). The glutathione content is expressed in μmol g^{-1} FW.

2.1.3. Assessments of Lipid Peroxidation (MDA)

The lipid peroxidation level was determined by the content of MDA [4]. Wheat roots (0.5 g) were ground with distilled water (3 mL). Then 3 mL of 20% trichloroacetic acid was added to the homogenate and centrifuged (10,000 *g* for 10 min). Then supernatant (2 mL) was mixed with 0.5% thiobarbituric acid (2 mL) prepared in 20 % trichloroacetic acid and was heated (100 °C for 30 min), and quickly cooled. The optical density of supernatant was measured at 532 nm and 600 nm using SmartSpecTM Plus spectrophotometer (Bio–Rad, Hercules, CA, USA). MDA concentration was 180 calculated using an extinction coefficient of 155,000 L cm^{-1} moL^{-1} and was expressed as nmoL g^{-1} FW.

2.1.4. AsA Content

The determination of ascorbic acid was carried out according to [6]. A weighed portion of plant material (1 g of roots for wheat seedlings) is homogenized with 10 mL of 2% metaphosphoric acid. The homogenate is transferred to a 50 mL volumetric flask. The volume is brought to the mark with 2% HPO_3 and 0.21 M Na_3PO_4, taken in a ratio of 3:2 (V/V, pH 7.3–7.4). The extract is centrifuged for 15 min at 3000 rpm, the extinction of the solution is measured on a spectrophotometer at 265 nm against the standard—the above solutions of HPO_3 and Na_3PO_4, taken in the same ratio. The molar extinction coefficient for ascorbic acid at 265 nm and pH = 6.8 and above equal to 1.65–1.655 × 10^4. The ascorbic acid content is expressed in μg g^{-1} FW.

2.1.5. Determination of Glutathione Reductase Activite

Assessment of GR (EC: 1.6.4.2) activity is based on the ability of the enzyme to catalyze the reduction of oxidized glutathione using NADPH as a reducing agent; during the analysis, the change

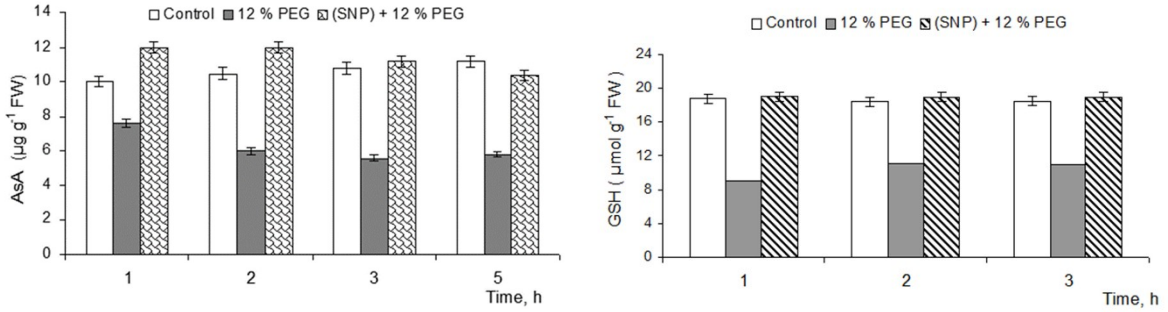
in the absorption of the solution during the formation of $NADP^+$ is measured [5]. For this, 0.25 g of the root weighed portion was ground in liquid nitrogen, then 0.75 mL of buffer containing 680 mg of K_2HPO_4, 4 g of polyvinylpyrrolidone, 3 mg of Na-EDTA, 280 μL of Triton-X dissolved in 100 mL of distilled water after which the mixture was centrifuged for 10 min at 12,000 *g*, the resulting supernatant was used in the work. The reaction mixture contained 50 μL of extract, 1.91 mL of measurement buffer containing 6.05 g of Tris-HCl and 146 mg of Na-EDTA dissolved in 500 mL of distilled water (pH 8.0) and 20 μL of a 0.4% solution of NADPH (Sigma-Aldrich, USA). The reaction in the mixture was initiated by introducing 20 μL of a 3% solution of oxidized glutathione (Sigma-Aldrich, USA). The control sample contained all introduced components, except for oxidized glutathione. The GR activity was measured every second for 5 min at a wavelength of 340 nm. When calculating the activity of glutathione reductase, we used the extinction coefficient for NADPH equal to 6.2 mM^{-1} cm^{-1} and expressed in units of mmol mg^{-1} (protein) min^{-1}. The protein content in leaf extract was determined by the protein-dye binding method of Bradford [7].

3. Results and Discussion

3.1. Subsection

3.1.1. Effect of 200 μM SNP on Pool AsA and GSH in the Roots of Wheat Plant under Drought Conditions

Analysis of the content of ascorbate and GSH showed that moisture deficit leads to a significant drop in the level of these most important non-enzymatic antioxidants throughout the exposure time (Figure 1).



(**a**) (**b**)

Figure 1. Effect of 200 μM SNP on the content of ascorbate (**a**) and glutathione (**b**) in the roots of 4-day-old wheat plants during exposure to 12% PEG. Different letters indicate a significant difference between the means at the probability level of $p < 0.05$.

Such reactions of plants to a violation of the water regime are quite natural, since oxidative stress caused by the action of 12% PEG depletes the pool of ascorbate and glutathione. SNP pretreatment practically prevented stress-induced depletion of the GSH pool (Figure 1b) and promoted not only the maintenance, but also the additional accumulation of ascorbate in them, which is observed in the first hours of exposure to stress (Figure 1a). This indicates that nitric oxide is able to regulate the content of ascorbate and glutathione under stress conditions, which is reflected in the stabilization of the antioxidant system and plant growth as a whole (data not shown). It is well known that ascorbate and glutathione play an important role in the regulation of the plant cell cycle–mitosis [8], which in general determines the important role of these antioxidants in the regulation of plant growth and development.

3.1.2. Effect of 200 μM SNP on Activity GR and Content MDA in the Roots of Wheat Plant under Drought Conditions

Exposure to drought modeled by 12% PEG, along with a drop in glutathione, leads to a significant activation of GR, a key enzyme involved in the recovery process GSSG to GSH (Figure 2a).

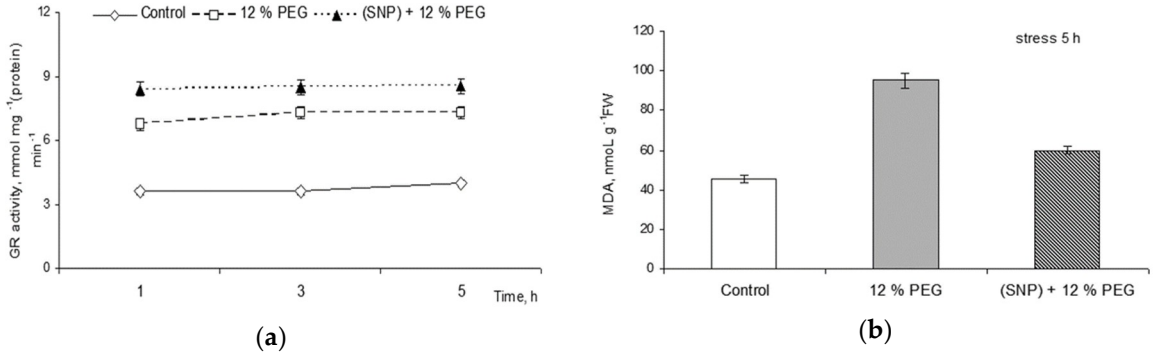


Figure 2. Effect of 200 μM SNP on the activity GR (**a**) and content MDA (**b**) in the roots of 4-day-old wheat plants during exposure to 12% PEG. Different letters indicate a significant difference between the means at the probability level of $p < 0.05$.

The pretreatment contributes to the additional activation of the GR (Figure 2a) this is reflected in the level of GSH in them (Figure 1b). An important indicator of the physiological state of a cell is the indicators of the integrity of membrane structures, the effect of stress caused a pronounced damaging effect, which was judged by a significant increase in the MDA content (Figure 2b). Seedling pretreatment with SNP contributed to a decrease in the negative effect of drought simulated by PEG on the integrity of membrane structures, as evidenced by data on a decrease in the level of stress-induced MDA accumulation; this is another argument in favor of using nitric oxide to increase wheat resistance to moisture deficiency.

4. Conclusions

Thus, an important contribution to the formation of SNP-induced resistance of wheat plants to the action of 12% PEG is made by its ability to regulate the content of glutathione and ascorbate, which, in general, leads to stabilization of the state of cell membrane structures and normalization of plant growth. The results obtained indicate the protective effect of 200 μM SNP on wheat seedlings under drought, thereby expanding the spectrum of manifestations of the protective effect of nitric oxide in plants.

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Conflicts of Interest: The authors declare no conflict of interest.

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