UDC 581.19 + 577.161.3

EFFECT OF SODIUM NITROPRUSSIDE ON ACTIVITIES OF ANTIOXIDANT AND GLUTATHIONE-RELATED ENZYMES IN LEAVES OF MAIZE SEEDLINGS

Y. V. VASYLYK[1,*], N. M. SEMCHUK[1], OK. V. LUSHCHAK[2]

[1]*Department of Biochemistry and Biotechnology,*
[2]*Botanical Garden, Precarpathian National University named after Vassyl Stefanyk,*
57 Shevchenko Str., 76025, Ivano-Frankivsk, Ukraine
**e-mail for correspondence: juliavasylyk@ukr.net*

Sodium nitroprusside was used as nitric oxide (•NO) donor to investigate effects of •NO on the activity of antioxidant enzymes in leaves of maize seedlings. Seeds of maize hybrid Kharkivskyj 195 MB (Zea mays L.) were used for the experiments. Leaves of 10-day old seedlings were cut off and transferred into round-bottomed flasks with distilled water or 0.1, 0.5 and 1.0 mM sodium nitroprusside solution (SNP). They were incubated for 24 h at constant shaking at 150 rpm and illumination with an 18 W fluorescent light, giving 800 lux of light intensity (16/8 h day/night regime and 26°C). The equimolar concentrations of potassium hexacyanoferrate (II) (PCF), were used as an additional control to SNP, since it has a chemical structure similar to SNP but lacks the ability to produce •NO. It was shown that treatment of leaves with SNP did not affect catalase activity, but PCF exposure enhanced it by 38-49%. The activity of ascorbate peroxidase was increased by 44-60% in leaves treated with all three concentrations of PCF, while only 0.1 and 0.5 mM of SNP enhanced it by ~60%. Guaiacol peroxidase activity was substantially suppressed by 71-80% and 45-69% in leaves incubated with both effectors, PCF and SNP, respectively. Potassium hexacyanoferrate (II) at all concentrations used did not affect glutathione-S-transferase activity in the leaves of maize seedlings, while 0.5 and 1.0 mM SNP increased it by 18 and 28%, respectively. Glutathione reductase activity was higher in leaves treated with 0.1 and 1.0 mM PCF by 16 and 39%, respectively, whereas 0.5 mM PCF did not change it. At the same time, SNP at all concentrations used increased GR activity by 40-60%. The increase of enzyme activity might result from mild nitrosative/oxidative stress induced by •NO donor, and/or to some extent by iron and cyanide ions, which released at SNP decomposition.

Keywords: Zea mays, maize seedlings, antioxidant enzymes, nitric oxide.

Introduction. Nitric oxide donors are widely used to study •NO role in living organisms, particularly in plants. Some of them, such as S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), and sodium nitroprusside (SNP), release •NO during decomposition. The latter is broadly used for investigation of •NO effects on different biochemical parameters of plants (Floryszak-Wieczorek et al., 2006; Bethke et al., 2006). It is known that exogenously added •NO in low levels acts as an antioxidant and delays programmed cell death in barley aleuron cells (Beligni et al., 2002). It also decreased oxidative injury induced by drought and UV-B radiation in wheat seedlings (Tian and Lei, 2007). Pre-treatment of roots with •NO increased the activity of leaf superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase in citrus plants (Tanou et al., 2009).

This work aimed to investigate the effect of •NO-donor sodium nitroprusside on the activity of antioxidant and associated enzymes in the leaves of maize seedlings and to compare it with effects of potassium hexacyanoferrate (II), which has a chemical structure similar to SNP, but lacks the ability to produce •NO.

Materials and methods. Seeds of maize hybrid Kharkivskyj 195 MB (*Zea mays* L.) were used for the experiments. All chemicals were obtained from "Sigma" (USA), "Fluka" (Germany). Other chemicals were obtained from the local suppliers (Russia, Ukraine) of the highest purity available. Maize seeds were germinated in moist chambers during 5 days. The germinated seeds with the same length of roots were transferred in Hoagland solution (Hoagland and Arnon, 1950) and grown for 5 days at 6700 lux of light intensity, 16/8 h day/night regime and 26°C.

Experimental design. Leaves of 10-day old seedlings were cut off and transferred into round-bottomed flasks (Arasimowicz-Jelonek et al., 2011) with distilled water or 0.1, 0.5 and 1.0 mM sodium nitroprusside solution (SNP, $Na_2[Fe(CN)_5NO]$). They were incubated for 24 h at constant shaking at 150 rpm and illumination with an 18 W fluorescent light, giving 800 lux of light intensity (16/8 h day/night regime and 26°C) (Lyn D. and Williams H., 2003). The equimolar concentrations of potassium hexacyanoferrate (II) (PCF, $K_4[Fe(CN)_6]$, were used as an additional control to SNP, since it has a chemical structure similar to SNP but lacks the ability to produce •NO (Bethke et al., 2006).

Determination of enzyme activity and protein content. Leaves of maize seedlings were ground in liquid nitrogen and stored until use. Leaves powder was supplemented 1:10 (w/v) with 50 mM potassium-phosphate (KPi) buffer (pH 7.0) containing 0.5 mM EDTA and 1.0 mM phenylmethyl-sulfonylfluoride. The extraction buffer for APX contained 1 mM ascorbic acid. The homogenates were centrifuged (Eppendord 5415R, Germany) at 13200 g during 15 min at 4 °C. The resulted supernatants were used for measurement of enzyme activity and protein concentration.

Catalase (EC 1.11.1.6) activity was measured spectrophotometrically at 240 nm (Aebi, 1984). The activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was monitored by following the decrease of absorbance at 290 nm wavelength (ε = 2800 M^{-1} cm^{-1}) (Chen and Asada, 1989). Guaiacol peroxidase (GuPX; EC 1.11.1.7) activity was assayed spectrophotometrically following the increase in absorbance at 470 nm due to guaiacol oxidation (ε = 26.6 mM^{-1} cm^{-1}) (Ali et al., 2005). Glutathione-S-transferase (GST; EC 2.5.1.18) activity was measured by monitoring the formation of adduct between GSH and 1-chloro-2,4-dinithrobenzene at 340 nm (ε = 9.6 mM^{-1} cm^{-1}) (Lushchak et al., 2005). Glutathione reductase (GR; EC 1.6.4.2) activity was determined as the decrease in absorbance at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) due to oxidation of reduced NADPH (Lushchak et al., 2005). One unit of enzymatic activity is defined as the amount of the enzyme consuming 1 μmol of substrate or generating 1 μmol of product per minute; the activities were expressed as international units (or milliunits) per milligram of protein. Protein concentration was determined with Coomassie brilliant blue G-250 according to the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Statistics. All experiments were carried out in three analytical and three independent biological repeats. Experimental data are expressed as mean ± SEM, and statistical testing was carried out with ANOVA followed by Dunnett's test.

Results and discussion. *Hydrogen peroxide-detoxifying enzymes.* Hydrogen peroxide (H_2O_2) is one of reactive oxygen species which also is a signaling molecule in plants and other living organisms (Lushchak, 2014). In plants H_2O_2 is eliminated via different mechanisms including enzymes, such as catalase, ascorbate peroxidase (APX), and guaiacol peroxidase (GuPX) (Gill and Tuteja, 2010). In our study the activity of catalase in control leaves of maize seedlings consisted of 19.9 ± 0.4 units/mg protein. It was not changed in leaves treated by SNP, at any concentration used, but PCF exposure enhanced it by 38-49% (Fig. 1A). It can be suggested, that •NO released at SNP decomposition either attenuated, or nullified effects of compounds, released at SNP and PFC decomposition. In our study the activity of APX in control leaves of maize seedlings consisted of 182.7 ± 13.9 units/mg protein. It was increased by 44-60% in leaves treated with all three concentrations of PCF, while only 0.1 and 0.5 mM of SNP enhanced it by ~60% (Fig. 1B). Similar results were observed in detached Arabidopsis leaves, incubated with SNP (Semchuk et al., 2011).

It was supposed that •NO-donors could increase the activity of abovementioned enzymes by the stimulation of H_2O_2 producing system(s) (Ederli et al., 2009).

Guaiacol peroxidase (GuPX) also uses H_2O_2 as a co-substrate to oxidize different compounds, particularly phenols, and the enzyme is supposed to be a stress-inducible one (Ghamsari et al., 2007). In our experiments, GuPX activity in control leaves of maize seedlings consisted of 1.16 ± 0.07 units/mg protein. Interestingly, GuPX activity was substantially suppressed by 71-80% and 45-69% in leaves incubated with both effectors, PCF and SNP, respectively (Fig. 1C), i.e. SNP decreased the GuPX activity to smaller extent than PCF. It is known, that •NO can inhibit peroxidases by binding to their prosthetic heme group, resulting in heme nitrosylation which, in turn, prevents interaction of H_2O_2 with the iron ions (Ferrer and Barcelo, 1999). However, in our experiments it really protected GuPX against inhibition by PCF.

Glutathione-related enzymes. Glutathione is inevitable component of virtually all living organisms (Lushchak, 2012). It is used for detoxification of reactive species either via direct interaction with them, or as a cofactor of specific detoxifying enzymes, such as glutathione-S-transferase (GST) and glutathione reductase (GR) (Gill and Tuteja, 2010; Lushchak, 2012). The activity of GST in control leaves of maize seedlings consisted of 89.1 ± 2.2 units/mg protein. Potassium hexacyanoferrate (II) at all concentrations used did not affect GST activity in the leaves of maize seedlings, while 0.5 and 1.0 mM SNP increased it by 18 and 28%, respectively (Fig. 2A). Previous studies demonstrated that •NO stimulated the expression of GST gene in soybean (Delledonne et al., 1998) and tobacco (Durner et al., 1998) plants exposed to pathogens and this mechanism could be involved in our case. So it can be that •NO enhanced GST activity via synthesis of new enzyme molecules. The activity of GR in control leaves of maize seedlings consisted of 34.0 ± 1.1 units/mg protein. It was higher in leaves treated with 0.1 and 1.0 mM PCF by 16 and 39%, respectively, whereas 0.5 mM PCF did not change it (Fig. 2B).

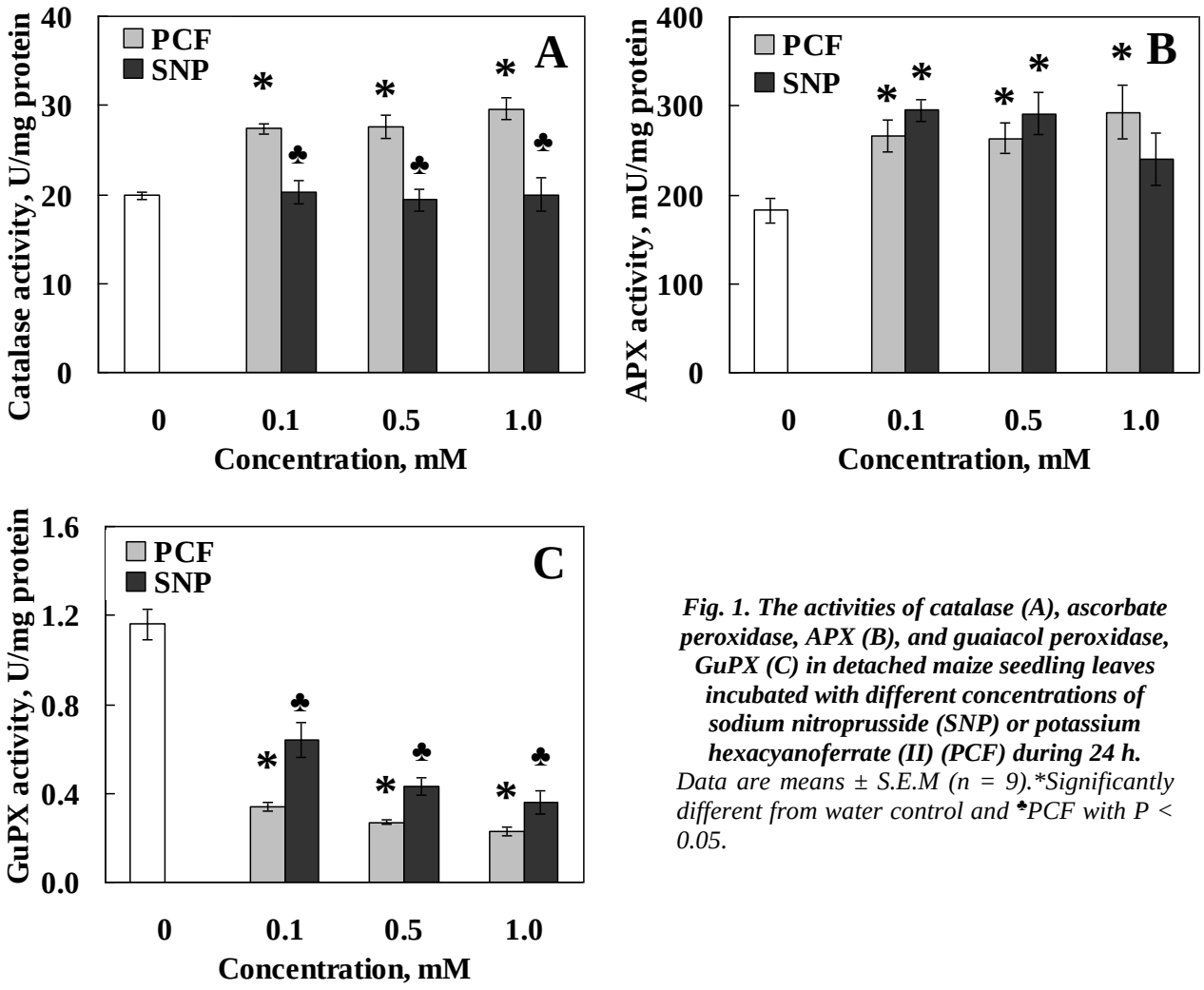

Fig. 1. The activities of catalase (A), ascorbate peroxidase, APX (B), and guaiacol peroxidase, GuPX (C) in detached maize seedling leaves incubated with different concentrations of sodium nitroprusside (SNP) or potassium hexacyanoferrate (II) (PCF) during 24 h.
*Data are means ± S.E.M (n = 9).*Significantly different from water control and ♣PCF with P < 0.05.*

At the same time, SNP at all concentrations used increased GR activity by 40-60%. These data well confirm the idea concerning the protective effect of both compounds used, SNP and PCF, against free radicals in the leaves of maize seedlings via increase of the activities of antioxidant and glutathione-related enzymes. Uchida and colleagues (2002) showed that pre-treatment of rice seedlings with SNP during two days augmented GR activity. SNP treatment also increased GR activity under stress induced by NaCl treatment of the leaves of citrus plants (Tanou et al., 2009).

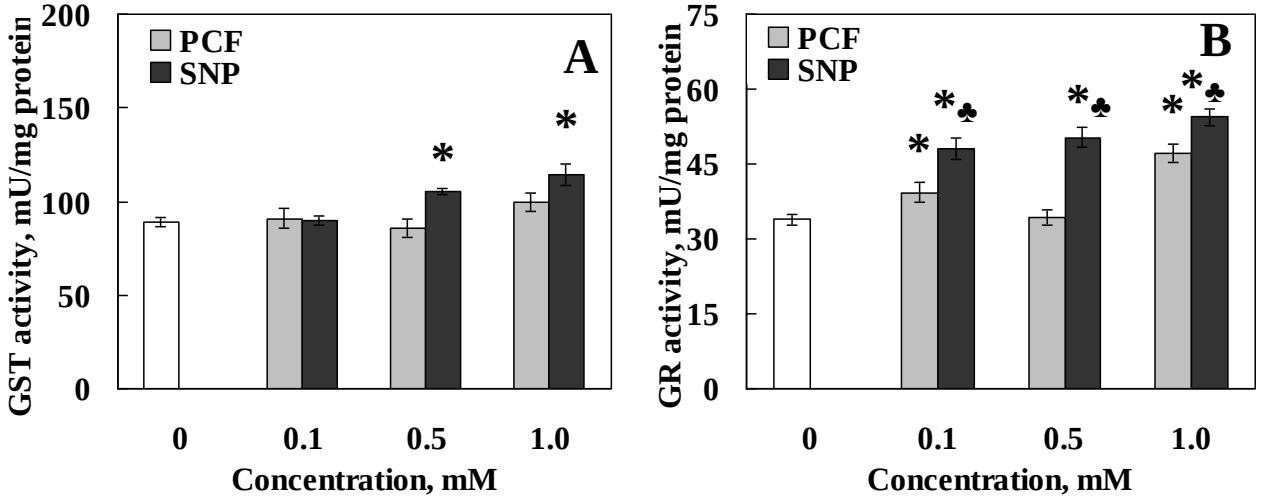

Fig. 2. The activities of glutathione-S-transferase, GST (A) and glutathione reductase, GR (B) in detached maize seedling leaves incubated with different concentrations of sodium nitroprusside (SNP) or potassium hexacyanoferrate (II) (PCF) during 24 h.
*Data are means ± S.E.M (n = 9).*Significantly different from water control and ♣PCF with P < 0.05.*

It seems that treatment of different plants with SNP enhances their antioxidant potential via increment of activities of antioxidant and associated enzymes.

Conclusions. It can be concluded that both compounds used, SNP and PCF, similarly affected investigated parameters in detached leaves of maize seedlings, but in the case with SNP these effects were more pronounced. The latter could be attributed to nitric oxide released at SNP decomposition due to several reasons. The first, •NO, as a signal molecule, might enhance the expression of genes encoding antioxidant enzymes measured here. The second, •NO might influence as a direct antioxidant and prevent protein oxidation. Therefore, •NO is supposed to affect leaves either directly as the antioxidant, or via diverse regulatory pathways and these •NO effects can ameliorate plant potential to survive detrimental environmental conditions.

References:

1. Aebi H., Catalases, in: H.U. Bergmeyer (Ed.) // Meth. Enzym. Anal. – 1984. – V. 2.– P. 673 – 684.
2. Ali M.B., Hahn E.J., Paek K.Y. Effect of temperature on oxidative stress defense systems, lipid peroxidation and lipoxygenase activity in *Phalaenopsis* // Plant Physiol. Biochem. – 2005 – V. 43. – P. 213 – 223.
3. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Kosmala A. Are nitric oxide donors a valuable tool to study the functional role of nitric oxide in plant metabolism? // Plant Biology. – 2011. – V. 13(5) . – P. 747 – 756.
4. Beligni M., Fath A., Bethke P.C., Lamattina L., Jones R. Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleuron cells // Plant Physiol. – 2002. – V. 129. – P. 1642 – 1650.
5. Bethke P.C., Libourel I.G., Reinöhl V., Jones R.L. Sodium nitroprusside, cyanide, nitrite, and nitrate break *Arabidopsis* seed dormancy in a nitric oxide-dependent manner // Planta. – 2006. – V. 223. – P. 805 – 812.
6. Bradford M.M. A rapid and sensitive method for the quantitation of protein-dye binding // Anal. Biochem. – 1976. – V. 72. – P. 248 – 254.
7. Chen G.X., Asada K. Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties // Plant Cell Physiol. – 1989 – V. 30. – P. 987 – 998.
8. Delledonne M., Xia Y., Dixon R.A., Lamb C. Nitric oxide functions as a signal in plant disease resistance // Nature. – 1998. – V. 394. – P. 585 – 588.
9. Durner J., Wendehenne D., Klessig D.F. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose // Proc. Natl. Acad. Sci. USA. – 1998. – V. 95. – P. 10328 – 10333.
10. Ederli L., Reale L., Madeo L., Ferranti F., Gehring C., Fornaciari M., Romano B., Pasqualini S. NO Release by nitric oxide donors in vitro and in planta // Plant Physiol. Biochem. – 2009. – V. 47. – P. 42 – 48.
11. Ferrer M.A., Barcelo A.R. Differential effects of nitric oxide on peroxidase and H_2O_2 production by the xylem of *Zinnia elegans* // Plant Cell Environ. – 1999. – V. 22. – P. 891 – 897.
12. Floryszak-Wieczorek J., Milczarek G., Arasimowicz M., Ciszewski A. Do nitric oxide donors mimic endogenous NO-related response in plants? // Planta. – 2006. – V. 224. – P. 1363 – 1372.
13. Ghamsari L., Keyhani E., Golkhoo S., Kinetics properties of guaiacol peroxidase activity in *Crocus sativus* L. corn during rooting // Iran. Biomed. – 2007. – J. 11. – P. 137 – 146.
14. Gill S.S., Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants // Plant Physiol. Biochem. – 2010. – V. 48. – P. 909 – 930.
15. Hoagland D.R, Arnon D.I. The water-culture method for growing plants without soil // California Agricultural Experimental Station Circular. Berkeley: CA University California. – 1950.
16. Lushchak V.I. Glutathione homeostasis and functions: potential targets for medical interventions // J. Amino Acids. – 2012. – V. 2012. – P. 1 – 26.
17. Lushchak V.I. Free radicals, reactive oxygen species, oxidative stress and its classification // Chem. Biol. Interact. – 2014. – V. 224. – P. 164 – 175.
18. Lushchak V.I., Bagnyukova T.V., Husak V.V., Luzhna L.I., Lushchak O.V., Storey K.B. Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues // Int. J. Biochem. Cell Biol. – 2005. – V. 37. – P. 1670 – 1680.
19. Lyn D. and Williams H., A chemist's view of the nitric oxide story. // Org. Biomol.Chem. – 2003. – V. 1. – P. 441-449.
20. Semchuk N.M., Vasylyk Yu.V., Kubrak O.I., Lushchak V.I. Effect of sodium nitroprusside and S-nitrosoglutathione on pigment content and antioxidant system of tocopherol-deficient plants of *Arabidopsis thaliana* // Ukr. Biokhim. Zh. – 2011. – V. 83. – P. 69 – 79.
21. Tanou G., Molassiotis A., Diamantidis G. Hydrogen peroxide- and nitric oxide-induced systemic antioxidant prime-like activity under NaCl-stress and stress-free conditions in citrus plants // J. Plant Physiol. – 2009. – V. 166. – P. 1904 – 1913.
22. Tian X.R., Lei Y.B. Physiological responses of wheat seedlings to drought and UV-B radiation. Effect of exogenous sodium nitroprusside application // Russ. J. Plant Physiol. – 2007. – V. 54. – P. 676 – 682.
23. Uchida A., Jagendorf A.T., Hibino T., Takabe T. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice // Plant Sci. – 2002. – V. 163. – P. 515 – 523.

ВПЛИВ НІТРОПРУСИДУ НАТРІЮ НА АКТИВНІСТЬ АНТИОКСИДАНТИХ ТА ГЛЮТАТІОН-ЗВ'ЯЗАНИХ ФЕРМЕНТІВ У ЛИСТКАХ ПРОРОСТКІВ КУКУРУДЗИ

Ю. В. Василик[1,*], Н. М. Семчук[1], Ок. В. Лущак[2]

[1]*Кафедра біохімії та біотехнології,*
[2]*Ботанічний сад, Прикарпатський національний університет імені Василя Стефаника,*
вул. Шевченка, 57, 76025, Івано-Франківськ, Україна
**e-mail для листування: juliavasylyk@ukr.net*

Нітропрусид натрію використаний як донор оксиду азоту (•NO) для дослідження впливу •NO на активність антиоксидантних ферментів у листках проростків кукурудзи. Насіння кукурудзи (Zea mays L.) гібриду Харківський 195 МВ було використано для експериментів. Листки (1 г) десятиденних проростків кукурудзи зрізали та переносили у круглодонні колби, які містили різні концентрації (0,1, 0,5 та 1,0 мМ) нітропрусиду натрію (НПН) у дистильованій воді. Листки інкубувались протягом 24 год при постійному перемішуванні 150 об/хв та цілодобовим освітленням флуоресцентними лампами (18 Вт) при інтенсивності освітлення 15 мкмоль фотонів $м^{-2}$ $с^{-1}$. Фериціанід калію (ФЦК) використовувався як додатковий контроль, оскільки він має хімічну структуру подібну до нітропрусиду натрію, однак не виділяє оксиду азоту. Було показано, що обробка НПН не впливала на активність каталази, проте обробка ФЦК підвищувала її активність на 38-49%. Активність аскорбатпероксидази була вищою на 44-60% в листках, оброблених трьома концентраціями ФЦК, в той час як лише 0,1 та 0,5 мМ НПН підвищувала її активність на ~60%. Активність гваяколпероксидази була значно знижена на 71-80% та 45-69% в листках, інкубованих з обома речовинами, ФЦК та НПН, відповідно. Всі використані концентрації фериціаніду калію не впливали на активність глютатіон-S-трансферази, в той час як обробка 0,5 та 1,0 мМ нітропрусиду натрію підвищувала активність даного ферменту на 18 та 28% відповідно. Експозиція проростків до 0,1 та 1,0 мМ ФЦК підвищувала активність глютатіонредуктази на 16 та 39%, однак обробка 0,5 мМ ФЦК не призводила до її змін. В той же час, всі концентрації нітропрусиду натрію підвищували її активність на 40-60%. Підвищення ферментативної активності могло бути викликане слабким нітрозитивним/оксидативним стресом, спричиненим донором •NO, або частково іонами заліза і/або ціаніду, які вивільняються в процесі розкладу нітропрусиду натрію.

Ключові слова: Zea mays, антиоксидантні ферменти, оксид азоту.

Одержано редколегією 20.11.2014