Australian Journal of Crop Science AJCS

AJCS 15(01):82-87 (2021) ISSN:1835-2707
doi: 10.21475/ajcs.21.15.01.2805

Nitric oxide as a way to mitigate copper (Cu) toxicity in the emergence and initial growth of maize seedling (*Zea mays* L.)

Ana Ecidia de Araújo Brito[1]*, Gerson Diego Pamplona de Albuquerque[1], Juscelino Gonçalves Palheta[1], Jessica Suellen Silva Teixeira[1], Diana Jhulia Palheta de Sousa[1], Keila Beatriz Silva Teixeira[1], Glauco André dos Santos Nogueira[1], Cândido Ferreira de Oliveira Neto[1], Ismael de Jesus Matos Viégas[2], Ricardo Shigueru Okumura[3]

[1]Agricultural Sciences Institute, Federal Rural University of Amazônia, CEP: 66077-530, Belém, Pará, Brazil
[2]Federal Rural University of Amazônia, Capanema Campus, Tv. Santa Cruz, 470 - Campinho, PA
[3]Federal Rural University of Amazônia, Parauapebas Campus, CEP: 68515-000, endereço: PA-275 s / n Zona Rural, Parauapebas, Pará, Brazil

Corresponding author: ecidiabrito@hotmail.com

Abstract

The aim of this study is to assess the effect of sodium nitroprusside-NPS (nitric oxide donor) on the emergence and initial growth of maize seedlings (*Zea mays* L.) subjected to different copper concentrations. The study followed a completely randomized design at 4 x 3 factorial arrangement, with 12 treatments and 8 repetitions, totally 96 trays with 25 seedlings per repetition. Seedlings were soaked in sodium nitroprusside (SNP) solution (0.75 and 150 µM - donor), sodium ferrocyanide (SF) (0.75 and 150 µM - compensator) and deionized water (control) on Germitest paper for 48 hours. Next, they were placed on sand saturated with $CuSO_4.5H_2O$ at concentrations of 0, 100 and 200 µM. The results showed that SNP doses were not capable of mitigating copper toxicity in seedling emergence, influencing emergence speed coefficient and prolonging the mean emergence time of plants subjected to copper concentrations of 100 µM and 200 µM. Copper concentrations rose the number of abnormal seedlings and had negative influence on plant biometrics and biomass. Copper concentration of 200 µM increased proline content in the roots. NPS and sodium ferrocyanide application had effect on emergence speed index, shoot and root dry mass and on proline content in the shoot and roots. This outcome highlights that such effects were caused by treatments related to release of cyanide found in sodium nitroprusside (SNP), rather than to nitric oxide (NO).

Keywords: seedlings; toxicity by metal; micronutrient.
Abbreviations: NO_Nitric oxide; RL_Root length; TL_Total length; SL_Shoot length; SDM_Shoot dry mass; RDM_Root dry mass; CRD_Completely randomized design; ESI_Emergence speed index; MET_Mean emergence time; ESC_Emergence Speed Coefficient; SNP_Sodium nitroprusside; SF_Sodium Ferrocyanide.

Introduction

The exploration of minerals and metals can change the natural contents of heavy metals in the environment. Mining practices generate waste that accounts for significant risk by adding its toxicity to the soil, water, plants and living organisms (Cunha, 2017).
Maize is an essential product for the Brazilian agriculture. It is grown in all regions, countrywide, in more than 2 million farms. This culture underwent changes in the last decades, with emphasis on its reduction as subsistence culture for small farmers and on its role in the efficient commercial agriculture sector due to geographical production and temporal displacement (Contini, 2019).
Copper (Cu) is a micronutrient essential for plants due its participation in plant metabolism and because it is a structural part of some enzymes (Gautam and Srivastava, 2016). High copper concentrations damage living beings. It has toxic effect on plant tissue and induces shortage of other basic nutrients because of antagonistic relationships (Taiz et al., 2017).
Plants grown in soils recording high copper content undergo biochemical stress, as well as drop in photosynthetic and respiration rates, which results in shorter roots and shoots, smaller root surface area and reduced biomass (Marques et al., 2018).
Nitric oxide (NO) is a signaling molecule involved in a wide range of responses to environmental stimuli. This molecule belongs to a family of reactive nitrogen species (RNS) because it has an unpaired electron. Therefore, it can interact with other reactive molecules such as Reactive Oxygen Species (ROS) and be eliminated from the cell. Besides, it can interact and change antioxidant enzymes and increase their activity (Arora et al., 2016; Salgado et al., 2017).
According to Santos (2009), the treatment with NO donor induced the activity of P-ATPase, V-ATPase and PPase, which

proves the hypothesis of its participation in the modulation of proton pumps. Nitric oxide is a messenger molecule; thus, in most cases, it responds to stress because of its interaction with phytohormones (Fan et al., 2014; Du et al., 2015; Sanz et al., 2015).

Copper toxicity in the physiological and biochemical attributes of plants was reported in studies by Apodaca et al. (2017), Marques et al. (2018) and Noreen et al. (2018). Contamination by copper led to reduced shoot and root dry mass production since growth is the final expression of all processes.

Studies have shown that the application of exogenous NO, in the form of sodium nitroprusside-SNP, improves plant tolerance to heavy metal-induced stress (Wang et al., 2013). These studies pointed out that the protective effect of SNP derived from NO release.

However, according to Silva et al. (2018), these effects were similar to those caused by the treatment with sodium nitroprusside. They were also observed in treatments with inactive sodium nitroprusside and ferrocyanide. This outcome indicates that the observed effects were associated with cyanide release by sodium nitroprusside.

Thus, the aim of the present study was to assess the mitigating effect of sodium nitroprusside, which is nitric oxide donor, based on variables related to the emergence, biometrics, biomass and proline contents in the leaves and roots of hybrid maize (variety K9606 VIP3) seedlings subjected to different copper concentrations.

Results and discussion

There was interaction between sodium nitroprusside-SNP and copper concentrations in the following variables: emergence speed index (ESI), root length (RL), root dry mass (RDM) and shoot dry mass (SDM), as shown in Tables 1 and 2.

Maize seedling emergence

Sodium nitroprusside-SNP doses did not affect seedlings' emergence parameters, but treatments added with copper had isolated effect on them. The treatment with 0µM of copper recorded mean emergence of 82.11% in comparison to treatments with 100 µM and 200 µM of copper, which have recorded 0% seedling emergence. There was 100% reduction in the emergence of seedlings subjected to treatments with 100 µM and 200 µM of copper in comparison to the treatment with 0µM (Table 1).

The drastic effect of heavy metals on seed emergence can be related to reduced activity of α and β amylases. This outcome compromises plant respiration and impairs growth in the embryonic and root axis (Pires et al., 2016). Therefore, the inhibition of these enzymes can point towards the toxicity mechanisms of sensitive plants exposed to these elements, which can work as indicators in contaminated environments given their fast response to damaging effects (Kong, 2013). Moreover, seedling emergence inhibition can be associated with reduced osmotic ability, with emergence medium potential and mainly with high copper concentrations (Ahsan et al., 2007).

Emergence speed index - ESI

The treatment with 0µM showed that some sodium nitroprusside-SNP doses mitigated copper toxicity effects in the treatment with 100 µM of copper, but it did not happen in treatments with 200 µM of copper. The highest mean (12.27%) was recorded for the treatment with 150 µM of SNP and 100 µM of copper. This number was increased to 21.6%, compared to the control. On the other hand, the lowest mean (8.21%) was observed for the treatment with 0 µM of SNP and 200 µM of copper compared to the control (0 µM of SNP and 200 µM of copper), which recorded 18.7% reduction in comparison to the control.

ESI delay due to exposure to Cu may have resulted from reduced nitrogen availability on the embryonic axis. It happens because of protein synthesis inhibition caused by lower availability of amino acids in the tegument (Karmous et. al., 2012).

However, some authors argue that the effect of nitric oxide donors actually derive from the nitric oxide itself. SNP was used to overcome dormancy in *Arabidopsis* seeds. Nevertheless, the authors have found that the effect resulted from cyanide in SNP (Bethke et al., 2006). One of the possible effects of cyanide would be O_2 availability for the oxidative pathway of pentose phosphate.

Emergence speed coefficient (ESC) and Mean Emergence Time (MET)

Sodium nitroprusside-SNP showed effect on emergence speed coefficient-ESC and copper had isolated effect on the treatment with copper concentration of 200 µM. The treatment with 0µM of copper recorded 34.33%, on average, which concerns 20.1% reduction in comparison to the treatment with 0 µM of copper (Table 1).

Variable mean emergence time-MET recorded longer mean emergence time in the treatment with copper concentration of 200 µM, which showed MET of 2.91%, whereas the treatment with 0 µM of copper recorded MET of 2.29%, which regards 27.3% increase in comparison to the treatment with 0 µM of copper (Table 1).

Germination delay can happen due to the protection role played by the tegument in seeds. It is capable of blocking and accumulating trace elements on its surface. Therefore, when the trace element is absorbed, it is deposited in the endosperm, which accounts for providing nutrients to the germination process; therefore, it can be translocated to the embryo (Sun and Luo, 2014).

According to Chaâbene et al. (2018), the beginning of root emergence requires high water content in the seeds, so that the metabolic activity necessary for germination can take place. Yet, seeds response to this hydration changes depends on their ability to control inner water level. Seed germination changes can be explained by changes in water absorption. Actually, cupric ions have toxic effect because they create an external osmotic potential to stop water capture in a way similar to that carried out by ions Na^+ and Cl^-.

Abnormal seedlings

Sodium nitroprusside-SNP doses did not mitigate the toxicity effect of copper in comparison to abnormal seedlings. However, there was isolated effect of copper concentrations in the treatments. Treatments with copper concentrations of 100 µM and 200 µM recorded means of 99.32% and 99.75%, respectively, in comparison to the treatment with 0µM of copper, which recorded mean of 16.01%. This outcome

Table 1. Total length (TL), shoot length (SL), proline in root (PR), emergence, emergence speed coefficient (ESC), abnormal seedlings, mean emergence time (MET) of maize seedlings treated with sodium nitroprusside (SNP), nitric oxide donor, sodium ferrocyanide (SFC), compensator and exposed to toxicity caused by copper.

Copper	TL	SDL	Proline in the root	Emergence	ESC	Abnormal seedlings	MET
(µM)	------ (cm)------		(µM Pro g^{-1} MS)	------------------------(%)------------------------			(day)
0	28.07 ± 2.00 a	5.72 ± 0.78 a	1.64 ± 0.18 c	82.11 ± 10.97 a	42.96 ± 4.22 a	16.01 ± 11.08 b	2.29 ± 0.28 c
100	6.29 ± 0.52 b	3.52 ± 0.45 b	4.33 ± 0.32 b	0.00 ± 0.00 b	40.32 ± 5.15 a	99.32 ± 1.48 a	2.51 ± 0.28 b
200	4.58 ± 0.31 c	2.53 ± 0.16 c	5.16 ± 0.23 a	0.00 ± 0.00 b	34.33 ± 1.97 b	99.75 ± 0.98 a	2.91 ± 0.16 a

Means followed by the same letter in the column did not differ from each other in the Tukey test at 5% probability level. Described values correspond to the means of eight repetitions and SD.

Table 2. Root length (RL), emergence speed index (ESI), shoot dry mass (SDM) and root dry mass (RDM) of maize seedlings treated with sodium nitroprusside (SNP), nitric oxide donor, sodium ferrocyanide (SFC), compensator and exposed to toxicity caused by copper.

Treatment	Copper	RL	ESI	SDM	RDM
	(µM)	(cm)		------(mg seedling^{-1}) ------	
	0	22.77 ± 1.19 ABa	10.10 ± 0.61 Ba	17.05 ± 2.61 Aab	68.85 ± 26.07 Aa
Water	100	3.45 ± 0.47 Ab	10.42 ± 0.75 ABa	19.30 ± 2.64 Aa	27.00 ± 3.51 Ab
	200	1.90 ± 0.16 Ac	9.10 ± 0.95 Aa	14.25 ± 2.64 Ab	33.10 ± 11.85 Ab
	0	21.66 ± 0.98 BCa	11.08 ± 0.77 ABa	18.10 ± 1.72 Aa	65.50 ± 21.65 Aa
SNP (0 µM) + SFC (150 µM)	100	2.72 ± 0.21Bb	10.04 ± 0.89 ABab	17.00 ± 3.40 ABab	30.30 ± 7.23 Ab
	200	1.98 ± 0.15 Ac	8.21 ± 0.61 Ab	14.10 ± 2.40 Ab	30.05 ± 4.03 ABb
	0	20.75 ± 1.24 Ca	11.68 ± 2.31 ABa	19.40 ± 1.86 Aa	68.95 ± 21.93 Aa
SNP (75 µM) + SFC (75 µM)	100	2.41 ± 0.19 Cb	9.37 ± 0.38 Ba	17.60 ± 2.69 ABab	34.45 ± 4.67 Ab
	200	2.08 ± 0.15 Ac	8.40 ± 0.21 Ab	15.30 ± 2.10 Ab	27.00 ± 3.81 ABc
	0	23.80 ± 1.84 Aa	11.63 ± 0.32 Aa	20.10 ± 2.93 Aa	83.35 ± 32.98 Aa
SNP (150 µM) + SFC (0 µM)	100	2.80 ± 0.27 Bb	12.27 ± 2.56 Aa	14.65 ± 3.82 Bb	30.30 ± 4.19 Ab
	200	2.04 ± 0.09 Ac	9.03 ± 0.85 Ab	14.40 ± 2.07 Ab	24.45 ± 4.29 Bc

Columns with different capital letters between treatments (water, SNP (0 µM) + SFC (150 µM), SNP (75 µM) + SFC (75 µM), SNP (150 µM) + SFC (0 µM) under the same copper concentration) and lowercase letters between copper concentrations (0, 100 and 200 µM under the same treatment) point out significant differences in the Tukey test at 5% probability level. Described values correspond to the means of five repetitions and SD.

Table 3. Proline in the shoot and proline in the root of maize seedlings treated with sodium nitroprusside (SNP), nitric oxide donor, sodium ferrocyanide (SFC), and compensators and exposed to toxicity caused by copper.

Treatment	Proline in the shoot	Proline in the root
	-------- (µM Pro g^{-1} MS)--------	
Water	3.51 ± 0.19 a	3.01 ± 0.49 b
SNP (0 µM) + SFC (150 µM)	3.48 ± 0.19 a	4.87 ± 0.64 a
SNP (75 µM) + SFC (75 µM)	2.92 ± 0.15 b	3.84 ± 0.44 a
SNP (150 µM) + SFC (0 µM)	2.95 ± 0.18 b	2.85 ± 0.38 b

Means followed by the same letter in the column did not differ from each other in the Tukey test at 5% probability level. Described values correspond to the means of eight repetitions and SD.

indicates 520.4% and 523% increase in treatments with copper concentrations of 100 µM and 200 µM, respectively, compared to 0 µM copper (Table 1).

The phytotoxic effect of copper leads to disturbance in cell development and differentiation, generating abnormal changes and reduces the rate of normal seedlings at germination (Pires et al., 2016). This effect evolves from membrane degeneration, reduced growth and development rate, lower uniformity and higher sensitivity to adversities, emergence reduction in the field and formation of abnormal seedlings, to loss of germination power (Marcos Filho, 2015).

Root Length (RL)

Variable showed that sodium nitroprusside-SNP only affected the treatment with 0 µM of copper, which recorded the highest mean root length (23.8cm) in the treatment with 150 µM of SNP and 0 µM copper – 4.6% increase in comparison to the control. The lowest mean root length (1.9cm) was observed for the treatment with copper concentration of 200 µM, which presented 91.7% root length reduction in comparison to the control treatment (Table 2). Enzyme activation block, reduced straight blocking of cell division and its influence on mitosis formation can explain the reduced root growth. Repression of protein synthesis and DNA replication can also block cell division (Moosavi et al., 2012).

NO action boosted root growth in the treatment with copper concentration of 0 µM. NO can be involved in the auxin signaling pathway, since it increases calcium level and activates protein kinases, which participates in the development of adventitious and lateral roots (Lanteri et al., 2006).

According to Ya'Acov and Haramaty (1996), NO acts in the phospholipid bilayer membrane and increases its fluidity. It can also act in relaxing cell wall and contribute to plant growth.

Based on Sarath et al. (2006), cyanide likely induces nitric oxide production at seed germination, which in its turn, aims proteins that favor cell elongation (root emergence) and growth (coleoptile extension).

The excess of this metal affects the root system, as well as shoot growth, and it induces disturbances in proteins and inhibits cell elongation due to increased plasmatic membrane permeability and cell wall lignification, which leads to plants with dwarfism symptoms (Yruela, 2009).

Shoot length (SL) and Total Length (TL)

Sodium nitroprusside-SNP doses did not influence shoot length (SL), but there was isolated effect of different copper concentrations. Copper concentration of 200 µM led to mean shoot length of 2.53cm, which concerns 55.8% reduction in comparison to the treatment with 0 µM of copper, which recorded mean shoot length of 5.72cm (Table 1).

There was no effect of sodium nitroprusside-SNP on total length-TL, but there was isolated effect of copper. The treatment with copper concentration of 200 µM presented mean total length of 4.58cm, which regards 83.7% reduction in comparison to the treatment with 0 µM of copper. It presented mean total length of 28.07cm (Table 1).

Copper excess can promote physiological, biochemical and morphoanatomical disturbances that can inhibit plant development (Gautam and Srivastava, 2016).

Besides reducing cell division, copper excess inhibits the activity of enzymes α-amylase and invertase, as well as affects nutrient accumulation mobilization and water absorption by the tissue (Sethy and Ghosh, 2013).

Root dry mass (RDM) and shoot dry mass (SDM)

The effect of sodium nitroprusside-SNP did not influence root dry mass-RDM in the treatment with 0 µM of copper. It showed that copper concentration of 200 µM accounted for reduced results in this variable.

The treatment with the following doses: 150 µM of SNP and 200 µM of copper, presented mean root dry mass of 24.45 mg; whereas, the control treatment recorded mean of 68.85 mg, which accounts for 64.5% reduction. On the other hand, the treatment with doses of 150 µM of SNP and 0µM of copper showed mean root dry mass of 83.35mg. It regards 21.1% increase in comparison to the control (Table 2).

There was sodium nitroprusside-SNP effect on shoot dry mass-SDM in the treatment with 0 µM of copper. The treatment with dose of 150 µM of SNP and 0 µM of copper recorded mean shoot dry mass of 20.10 mg, which concerns 17.9% increase in comparison to the control treatment. It presented mean shoot dry mass of 17.05mg. The treatment with 0 µM of SNP and 200 µM of copper showed the lowest mean (14.10mg), which is 17.3% reduction in comparison to the control (Table 2).

The phytotoxic effect of heavy metals depends on plant development stage. It can be featured by diminished total plant phytomass, shoot and root length, delayed development and lower chlorophyll concentration (Pinto, 2017).

Root length is a parameter widely used in bioassays applied to phytotoxicity (Simões et al., 2013). According to these authors, reduced mean length in lettuce can be related to lower cell division rates in the meristematic region, since this region accounts for root growth. This process influences root dry mass reduction.

Proline content

There was isolated effect of copper concentration in proline content in the roots. The treatment with copper concentration of 200 µM recorded mean proline content in the roots of 5.16 µM Pro g^{-1} MS, which means 214.68% increase compared to 0 µM of copper, which presented mean proline content in the roots of 1.64 µM Pro g^{-1} MS (Table 1).

There was sodium nitroprusside-SNP effect on proline content in the shoot, which decreased in treatments with dose of 75 and 150 µM of SNP. They recorded mean proline content in the shoot of 2.92 and 2.95 µM Pro g^{-1} MS; 17.05% and 16.07% reduction, respectively; in comparison to the control (Table 3).

Sodium nitroprusside-SNP had isolated effect on the proline content in the roots. It meant increased rates in the treatment with 0 µM dose of NPS, which presented mean proline content in the roots ~ 4.87 µM Pro g^{-1} MS, meaning 61.73% increase in proline content in the roots in comparison to the control treatment (Table 3).

Nitric oxide is related to auxin transport and higher levels of radical impairs in primary root growth (Fernández-Marcos et al., 2011). However, this radical plays a relevant role in the development of the apical meristem at appropriate levels.

Nowadays, most studies about the interaction between indole acetic acid-AIA and NO are concentrated in root responses; therefore, information about the shoot are scarce (Simontacchi et al., 2013). According to Fahad et al. (2015), NO can modulate the AIA level and control its degradation.

Proline can carry out different functions in response to abiotic stress in plants, such as osmoprotective response, with emphasis on the protection role of the membrane against damaging effects caused by the excess of heavy metals. Oftentimes, there are high contents of these substances under stress conditions (Gautam and Srivastava, 2016).

NO increases the activity of enzyme pyrroline-5-carboxylate synthase (P5CS), which participates in proline biosynthesis and inhibits the activity of enzyme proline dehydrogenase (PDH) that, in its turn, is involved in NO activity degradation (Fan et al., 2012).

NO in plants has antioxidant action and regulates the level and toxicity of reactive oxygen species (ROS), which are generated by different stress types (Sang et al., 2008). Apparently, NO rises the ascorbate levels and activates enzymes of the antioxidant system in cells (Zhang et al., 2008). The protective effect of the exogenous application of SNP has been attributed to the elimination of superoxide radicals (• O2-) and/or to the increased activity of antioxidant enzymes (Wang et al., 2010).

Materials and Methods

Experiment conduction

The experiment was carried out in the Laboratory of Top Plant Studies (EBPS) of the Agricultural Sciences Institute of Federal Rural University of Amazônia (UFRA). Seeds of hybrid maize K 9606 VIP3 were provided by the company SAAT SE & Co. KGaA and used in the experiment.

Seed treatment

Seeds were soaked in solution with sodium nitroprusside Na_2 [Fe $(CN)_5$ NO]$2H_2O$ (0. 75 and 150 µM), with nitric oxide donor, sodium ferrocyanide Na_4Fe $(CN)_6$ (0. 75 and 150 µM), as compensator, and deionized water (control), on Germitest paper for 48 hours.

Seeds were placed on trays filled with washed and autoclaved sand, dried in air circulating oven at 70°C. The sand saturated with copper concentrations of $CuSO_4.5H_2O$ (0, 100 and 200 µM) and deionized water (control) at 60% field capacity, 8-h photoperiod (8 hours of light and 16 hours of darkness) and temperature of 25°C ± 2 °C (RAS).

Analysis of emergence, biometrics, biomass and proline content

The counting of emerged seedling was daily performed from 4^{th} to the 7^{th} day after experiment implementation. The emergence criteria were proposed by Brasil (2009). It led to the formation of seedlings presenting normal essential structures (primary root, coleoptile and plumule).

According to Silva and Nakagawa (1995), the emergence was expressed in percentages and the formulation expressed by Edmond and Drapala (1958) were used to calculate mean emergence time (MET). The formulation proposed by Kotowski (1926) was used to calculate the emergence speed coefficient. The emergence speed index was calculated through the formulation proposed by Maguire (1962).

Mean shoot length (MSL) and root system (RS) were measured at the end of the germination test. Eight (8) repetitions, with 15 seedlings from each treatment, were used in the experiment. Their measurements were carried out in the seventh day after sowing, with the aid of a millimeter ruler , results were expressed in $cm.seedling^{-1}$ (Nakagawa, 1999).

Shoot dry mass (SDM) and root dry system (RDS) were calculated through the method based on circulating air oven at 70°C used to reach constant weight (Nakagawa, 1999). Proline concentrations were determined based on the methods described by Bates et al. (1973).

Study design and statistical analysis

The study followed a completely randomized design at 4 x 3 factorial arrangement. The 4 treatments consisted of (water, SNP (0 µM) + SFC (150 µM), SNP (75 µM) + SFC (75 µM), SNP (150 µM) + SFC (0 µM) at 3 different copper concentrations (0, 100 and 200 µM), totally 12 treatments, with 8 repetitions; 96 trays filled with 25 seeds per repetition. Data were subjected to the analysis of variance (ANOVA) with Tukey test at 5% probability, carried out in the SISVAr software.

Conclusion

Sodium nitroprusside (SNP) concentrations and nitric oxide (NO) donor were not capable of mitigating copper toxicity in the emergence of maize seedlings belonging to variety K9606VIP3. They did not influence the emergence speed coefficient and prolonged the mean emergence time of seedlings subjected to treatments with copper concentrations of 100 µM and 200 µM.

Copper concentrations of 100 µM and 200 µM raised the number of abnormal seedlings. It had negative influence on the biometrics and biomass of maize seedlings. The highest copper concentration (200 µM) increased the proline content in the roots of maize seedlings, with emphasis on the osmoprotector role against abiotic stress caused by the excess of this metal.

Sodium nitroprusside, nitric oxide donor, and sodium ferrocyanide application showed effect on emergence speed index, shoot and root dry mass and on the proline content in the shoot and roots. This outcome indicates that such effects are caused by treatments that can be related to the release of cyanide found in sodium nitroprusside-SNP, rather than by nitric oxide. In-depth studies are necessary to reach better conclusions about the herein observed effects.

Acknowledgement

We are grateful to *Capes - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior,* for granting the scholarships and for UFRA – Federal Rural University of Amazônia. EBPS-Biodiversity Studies in Top Plants Team.

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