



## Beneficial behavior of nitric oxide in copper-treated medicinal plants



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### HIGHLIGHTS

- Endogenous NO and ROS accumulation were inversely related.
- Selected amino acids in the roots were increased by SNP.
- NO induced regulation of phenolic metabolism for protection against Cu toxicity.
- SNP improved the vincristine, vinblastine and total alkaloid contents in Cu-treated plants.

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### ABSTRACT

Despite numerous reports implicating nitric oxide (NO) in the environmental-stress responses of plants, the specific metabolic and ionic mechanisms of NO-mediated adaptation to metal stress remain unclear. Here, the impacts of copper (Cu) and NO donor (SNP, 50 μM) alone or in combination on the well-known medicinal plant *Catharanthus roseus* L. were investigated. Our results showed that Cu markedly increased Cu<sup>2+</sup> accumulation, decreased NO production, and disrupted mineral equilibrium and proton pumps, thereby stimulating a burst of ROS; in addition, SNP ameliorates the negative toxicity of Cu, and CPTIO reverses this action. Furthermore, the accumulations of ROS and NO resulted in reciprocal changes. Interestingly, nearly all of the investigated amino acids and the total phenolic content in the roots were promoted by the SNP treatment but were depleted by the Cu + SNP treatment, which is consistent with the self-evident increases in phenylalanine ammonia-lyase activity and total soluble phenol content induced by SNP. Unexpectedly, leaf vincristine and vinblastine as well as the total alkaloid content (ca. 1.5-fold) were decreased by Cu but markedly increased by SNP (+38% and +49% of the control levels). This study provides the first evidence of the beneficial behavior of NO, rather than other compounds, in depleting Cu toxicity by regulating mineral absorption, reestablishing ATPase activities, and stimulating secondary metabolites.

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### 1. Introduction

Metal pollution of the biosphere has increased dramatically due to industrial and agricultural activities, increasing the potential risk to human health. Copper/Cu(II), one of the most dangerous intermediate metals, is an essential plant micronutrient and a

cofactor for many enzymes [1–3]. However, an excess amount of this metal is cytotoxic in most plants. As a redox-active metal, Cu can directly stimulate the overproduction of reactive oxygen species (ROS) through Fenton–Haber–Weiss reactions, resulting in alterations to the membrane composition, a loss of vital nutrients and destruction of the metabolic equilibrium [4–6]. To minimize the toxic effects of metals, plants have evolved an ROS-scavenging system composed of (non-)enzymatic antioxidants. In particular, in plants, phenolic compounds (non-enzymatic antioxidants) are effective ROS scavengers (antioxidant properties) and/or radical chain breakers (due to their metal-chelating capability) [7,8] by quenching oxidative free radicals. Nevertheless, data related to

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**Table 1**

The background values of nutrient elements, organic matter, and electric conductivity (EC) in the studied yellow-brown loam. Macro- and micro-nutrient values are in mg kg<sup>-1</sup>; Fe, Na and Al values are percentages. \*, %, \*\*, mS cm<sup>-1</sup>. The available N, P and K contents were 19.3, 10.4 and 58.5 mg kg<sup>-1</sup>, respectively.

Macronutrients						Micronutrients						Organic			
N	P	K	Ca	Mg	S	Mn	Zn	Mo	Cu	Fe	Na	Al	matter*	EC**	pH
39.6	813.4	235.1	403.2	197.3	2.6	165.7	43.5	0.37	20.6	2.4	1.1	6.7	2.3	2.8	6.5

**Table 2**

Impacts of foliar spray of various concentrations exogenous SNP (in μM) in *C. roseus* plants grown in pot culture with 30 mg kg<sup>-1</sup> Cu for 60 DAP. Data are shown as the means ± SDs (*n* = 7 for biomass and *n* = 5 for the other measures). Different lowercase letters indicate that the mean values are significantly different among the treatments at *P* < 0.05 according to Tukey's test. †, cm<sup>2</sup> cm<sup>-2</sup> plant<sup>-1</sup>; \*\*, cm shoot/root<sup>-1</sup>; \*\*\*, mg plant<sup>-1</sup> DW.

	Control	Cu	Cu + 10SNP	Cu + 25SNP	Cu + 50SNP	Cu + 100SNP	Cu + 200SNP	Cu + 400SNP
Leaf-area index <sup>†</sup>	16.3 ± 0.86 <sup>a</sup>	9.3 ± 0.57 <sup>cd</sup>	10.2 ± 0.61 <sup>c</sup>	11.8 ± 0.35 <sup>b</sup>	12.6 ± 0.69 <sup>b</sup>	10.1 ± 0.51 <sup>c</sup>	9.1 ± 0.96 <sup>cd</sup>	8.5 ± 0.71 <sup>d</sup>
Shoot length <sup>**</sup>	67.6 ± 2.69 <sup>a</sup>	43.7 ± 1.98 <sup>ef</sup>	45.0 ± 2.17 <sup>de</sup>	50.2 ± 2.72 <sup>c</sup>	56.4 ± 1.69 <sup>b</sup>	48.9 ± 1.62 <sup>cd</sup>	44.6 ± 1.61 <sup>e</sup>	40.1 ± 1.16 <sup>f</sup>
Root length <sup>**</sup>	23.3 ± 2.36 <sup>a</sup>	12.2 ± 1.11 <sup>de</sup>	14.7 ± 0.46 <sup>bc</sup>	17.1 ± 1.18 <sup>b</sup>	16.8 ± 0.96 <sup>bc</sup>	14.6 ± 0.77 <sup>cd</sup>	12.1 ± 1.15 <sup>de</sup>	10.2 ± 0.89 <sup>e</sup>
Shoot dry weight <sup>***</sup>	6.82 ± 0.64 <sup>a</sup>	3.35 ± 0.38 <sup>de</sup>	3.84 ± 0.12 <sup>cd</sup>	4.17 ± 0.15 <sup>cd</sup>	5.32 ± 0.50 <sup>b</sup>	4.61 ± 0.51 <sup>bc</sup>	3.54 ± 0.35 <sup>d</sup>	2.63 ± 0.33 <sup>e</sup>
Root dry weight <sup>***</sup>	1.58 ± 0.10 <sup>a</sup>	0.72 ± 0.07 <sup>de</sup>	0.96 ± 0.09 <sup>c</sup>	0.90 ± 0.07 <sup>c</sup>	1.19 ± 0.06 <sup>b</sup>	1.15 ± 0.04 <sup>b</sup>	0.86 ± 0.04 <sup>cd</sup>	0.67 ± 0.06 <sup>e</sup>

their accumulation under metal-induced stress are scarce. Moreover, amino acids, along with mineral homeostasis in plants, also contribute to the regulation of the adverse effects of metals [9–11]. To date, the mechanisms of action of phenolic compounds in metal-stressed plants and the effects of exogenous regulators [e.g., nitric oxide (NO)] on metal-induced changes to phenolic metabolites and related physiological/biochemical parameters have not been studied.

Plants have evolved mechanisms for coping with metal toxicity that is linked to certain stress-signaling molecules. For example, NO might directly remove ROS as an antioxidant [12–14] or promote metal accumulation in root cell walls by increasing pectin and hemicellulose levels in the whole plant [15]. NO may also be a stress-inducing agent. In addition, the protective effect of NO may depend on the supplied dose, treatment time, and selected species. However, in plants, NO is involved in elicitor-induced secondary metabolite biosynthesis, such as that of shikonin, catharanthine and artemisinin [16,17], and understanding its transduction pathways is necessary for optimizing commercial production of anticancer alkaloids. However, the specific mechanisms of NO in the regulation of secondary metabolites and the variation in the antioxidants in medicinal plants have not yet been determined [18]. Another detoxification mechanism used by plant cells involves the prevention of metal transport across the plasma membrane via the binding of the metal absorbed through the cell wall and detoxification of metals entering the cytoplasm by chelation and deposition inside vacuoles. H<sup>+</sup>-transporting adenosine triphosphatase (H<sup>+</sup>-ATPase), an important enzyme in the plasma membrane (PM) and tonoplast vesicles (TV), regulates the cellular secondary transportation system by producing a proton electrochemical gradient in abiotically stressed plants [19,20]. However, the effects of metals on proton pumps and their interactions in plants remain unclear.

Madagascar periwinkle (*Catharanthus roseus* L.), a highly studied and exploited medicinal plant and a popular ornamental plant, contains more than 100 monoterpenoid indole alkaloids (MIAs) that are used for the treatment of both malignant and non-malignant tumors and platelet (associated) disorders [21,22]. All parts of the plant are credited with hypoglycemic properties and are used to treat diabetes. Meanwhile, the anticancer alkaloids vincristine and vinblastine are primarily present in the shoots (leaves), and the antihypertensive alkaloid ajmalicine is found in the roots [23]. Periwinkle is also a good source of antioxidants for scavenging free radicals, and antioxidant metabolism under salt stress, water deficits, hormone induction and growth regulation has been studied in these plants [21,22,24]. However, the role of NO in adaptation to metal-induced metabolic changes remains unknown.

In addition, in Asia, the continuous shrinking of cultivable lands discourages cultivation of non-crop plants in cultivable lands, resulting in the use of metal-abused lands for the cultivation of non-edible plants, such as those with medicinal value. Therefore, it is important to determine whether periwinkle can be successfully cultivated in Cu-contaminated soils. Due to the importance of this plant, the aim of our study was to conduct the first investigation of the impacts of NO and Cu on antioxidant levels, phenolic metabolites, mineral absorption, and antitumor alkaloids in periwinkle plants. The results provide a deeper understanding of the mechanisms of NO-mediated reduction of Cu cytotoxicity in medicinal plants.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Healthy periwinkle seeds (PanAmerican Seed, TX, USA) were surface-sterilized with a 2% NaClO solution for 8 min and then thoroughly rinsed with distilled water followed by germination in raised seedbeds. To simulate Cu-polluted soil, pot-controlled experiments were conducted at Sichuan Agricultural University (SICAU), Chengdu, China. At 30 days after sowing, similarly sized vigorous seedlings were transplanted into plastic pots with holes in the bottom [20 cm (inner-diameter) × 25 cm (height); 2 plants per pot] that were filled with 8.0 kg of air-dried yellow-brown loam (see Table 1) and irrigated to attain a leachate fraction (LF) of approximately 0.15 (i.e., 15% LF). Based on an investigation of the Cu-contaminated soil in this region by Zhang et al. [25], 30 mg kg<sup>-1</sup> was selected as the soil Cu concentration. As a pre-test, 30 mg kg<sup>-1</sup> Cu (CuCl<sub>2</sub>·2H<sub>2</sub>O) and 0–400 μM sodium nitroprusside (SNP; Na<sub>2</sub>[Fe(CN)<sub>5</sub>NO]·2H<sub>2</sub>O) were selected for the determination of the growth parameters 60 days after planting (DAP). The results indicated that Cu impeded plant growth and biomass accumulation and that 50 μM SNP provided the most alleviating effect (+27% in Leaf-area index/LAI, +29/38% in length, and +59/65% in DW relative to Cu-treated plants). Therefore, 50 μM SNP was chosen for use in further tests (see Table 2). The experiment was performed in a growth chamber under controlled conditions: a 12-h day (6:00 am–6:00 pm); a photon flux density of ~400 μmol m<sup>-2</sup> s<sup>-1</sup> PAR at leaf level, which was supplied by cool white fluorescent tubes (L36W/840, Lumilux, Osram, Germany), day/night temperatures of 25/20 °C and a relative humidity of ~70%.

To investigate the effect of NO on the Cu-treated plants, four treatments with Cu<sup>2+</sup> (final doses of 0 and 30 mg kg<sup>-1</sup>) and SNP (0 and 50 μM) were employed: distilled water without SNP [control], distilled water with 50 μM SNP [SNP], 30 mg kg<sup>-1</sup> Cu without

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