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Effects of exogenous salicylic acid and nitric oxide on physiological characteristics of two peanut cultivars under cadmium stress

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Abstract

The interactive effects of salicylic acid (SA) and nitric oxide (NO) on alleviating cadmium (Cd) toxicity in peanut (*Arachis hypogaea* L.) were studied. Seedlings of two cultivars (Huayu 22 - a big seed type, and Xiaobaisha - a small seed type) were treated with 200 μM $CdCl_2$ without or with 0.1 mM SA or 0.25 mM sodium nitroprusside (SNP, an NO donor). Results show that the Cd exposure depressed the plant growth of both the cultivars but more of Huayu 22 than of Xiaobaisha. Exogenous SA and NO alleviated Cd toxicity in both the peanut cultivars: they improved growth, chlorophyll content, photosynthesis, and mineral nutrition. Furthermore, exogenous SA or NO decreased oxidative stress by increasing activities of antioxidant enzymes and content of non-enzymatic antioxidants. Besides, in roots and leaves of both the cultivars, exogenous SA and NO increased Cd accumulation in the cell wall and decreased Cd distribution to organelles. In particular, the effect of SA+SNP was most obvious.

Additional key words: antioxidants, *Arachis hypogaea*, chlorophyll, mineral nutrition, net photosynthetic rate.

Introduction

Cadmium is a ubiquitous toxic heavy metal in the environment and presents a potential threat to human health (Jarup and Akesson 2009, Wang *et al.* 2013b). As non-essential element for plants, Cd is one of the most dangerous heavy metals (Hassan *et al.* 2005, Meng *et al.* 2009). Upon exposure to Cd stress, plants show a growth retardation, leaf chlorosis, altered stomatal function, inhibition of photosynthesis, *etc*. (Xue *et al*. 2013). On the cellular level, Cd has numerous negative effects, such as membrane distortion, production of toxic metabolites, and reactive oxygen species (ROS; Hassan *et al.* 2005, Wang *et al.* 2013a). Therefore, it is necessary to find an approach to alleviating Cd toxicity on crop growth in Cd-contaminated fields.

Endogenous salicylic acid (SA) can participate in regulation of plant growth, development, and responses to environmental stresses (Senaratana *et al.* 2000). There are many studies indicating that exogenous SA influences a range of diverse processes, including seed germination, ion uptake and transport, membrane permeability, photosynthesis, and antioxidative ability under stress (Panda *et al.* 2007, Shi *et al.* 2009, Kazemi *et al.* 2010, Kang et al. 2013). Many studies have demonstrated that exogenous SA also ameliorates damaging effects of heavy metals, like cadmium (Guo *et al.* 2007), lead (Mishra and Choudhuri, 1999), and nickel (Kazemi *et al.* 2010) in different plant species. According to Guo *et al.* (2009), SA-induced Cd tolerance in plants is attributable to SA enhancement of antioxidant defence activities and to SA regulation of Cd uptake, transport, and distribution in plant organs.

Nitric oxide is a signalling molecule which plays

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Abbreviations: ASA - ascorbate; CAT - catalase; Cd - cadmium; Chl - chlorophyll; GSH - glutathione; MDA - malondialdehyde; $O_2^{\bullet-}$ - superoxide anion radical; POD - peroxidase; ROS - reactive oxygen species; SA - salicylic acid; SOD - superoxide dismutase; SNP - sodium nitroprusside; TF - translocation factor.

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important roles in a variety of physiological processes, such as germination, growth (Arasimowicz and Floryszak-Wieczorek 2007), iron availability (Lamattina *et al.* 2003), stomatal movement (Bright *et al.* 2006), cell differentiation, and programmed cell death (Pedroso and Durzan 2000). Moreover, it has been reported that NO can exert a protective effect against drought (Zhao *et al.* 2008), heat (Song *et al.* 2006), salt (Lopez-Carrion *et al.* 2008), and heavy metals (Arasimowicz and Floryszak-Wieczorek, 2007, Singh *et al.* 2008) stresses. In relation to heavy-metal stress, it was shown that exogenous NO reduces harmful effects of Cd toxicity in rice, barley, wheat, and perennial ryegrass (Singh *et al.* 2008, Xiong *et al.* 2009, Chen *et al.* 2010, Wang *et al.* 2013a).

Peanut is an important oil crop which accumulates significant concentrations of Cd (Shi *et al.* 2010). Excess Cd content in kernels possesses a threat to human health. Shi *et al.* (2010) demonstrated that significant differences exist among cultivars of peanut in terms of Cd tolerance. In this study, two peanut cultivars were compared and we studied the interactive effects of SA and NO (SNP, a donor of NO) on the responses of seedlings to Cd stress. The purpose of the present study was mainly to examine the possible protective roles of exogenous SA and NO against Cd-induced oxidative stress.

Materials and methods

Two cultivars of peanut (*Arachis hypogaea* L.) seeds were used in this experiment. Huayu 22 is a big seed type, and Xiaobaisha is a small seed type. The seeds were sterilized with 5 % (m/v) sodium hypochlorite for 15 min and washed extensively with distilled water, then germinated on moist filter paper in the dark at 28 °C for 3 d. Uniform sized peanut seedlings were transferred to plastic pots (volume 1 000 cm^3) filled with *Perlite* (six plants per pot at first) and watered with a half-strength Hoagland nutrient solution (Hoagland and Arnon 1950) for 7 d, then were watered with a full-strength Hoagland solution. Three-week-old uniform seedlings were transplanted to glass pots (three plants per pot) which contained 1 000 cm^3 of a fresh nutrient solution with 200 μM $CdCl_2$ either with or without 0.1 mM SA and 0.25 mM SNP or SA+SNP. The Hoagland solution was adjusted to pH 6.5 and renewed every two days. The treatments were arranged in a randomized block design with 5 replicates, giving a total of 40 containers. Plants were grown in a growth chamber at a 14-h photoperiod, a photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1} at the leaf level, day/night temperatures of 25/18 °C, and a relative humidity of 65 ± 5 %. After two weeks of growth, the plants were harvested. Roots and shoots were separated and washed with 5 mM $CaCl_2$ and then repeatedly washed with deionized distilled water. For determinations of plant dry matter, Cd and mineral element content, the plants were dried at 80 °C for 48 h. For enzyme activities determinations, fresh plant material was frozen in liquid nitrogen and stored at -70 °C (Wang *et al.* 2013a).

Leaf area was measured with an area meter (*CI-202*, *CID*, USA). Roots and shoots were separated and oven-dried at 105 °C for 30 min and then at 70 °C until the material reached a constant mass. The dried tissues were weighed and ground into powder for the determination of the concentrations of Cd and mineral elements which were measured by flame atomic absorbance spectrometry (*Shimadzu AA-6300*, Kyoto, Japan) after digestion with mixed HNO_3 and $HClO_4$ (3:1, v/v) (Wang *et al.* 2013a). Translocation factors (TFs) were calculated as: TF = [Cd] shoot / [Cd] root.

The content of chlorophylls (Chl) *a+b* was determined according to Arnon (1949). Fresh peanut leaves (0.1 g) were extracted with four volumes of 80 % (v/v) acetone until complete bleaching, and the extracted solution was analyzed spectrophotometrically (*Shimadzu UV-2450*, Kyoto, Japan) at 663 and 645 nm.

Net photosynthetic rate was monitored *in vivo* with a portable photosynthesis system (*LI-6400*, *LICOR*, Lincoln, NB, USA). Measurements were done in sunny and clear weather in the period between 09:00 and 11:00. All measurements were taken at a constant air flow rate of 500 μmol s^{-1}, a saturation PPFD of 1 200 μmol m^{-2} s^{-1}, an ambient CO_2 concentration of 350 cm^3 m^{-3}, and a temperature of 25 °C.

For the determination of H_2O_2 content, leaf tissue (0.2 g) was extracted with 3 cm^3 of 0.1 % (m/v) trichloro-acetic acid (TCA) in an ice bath and centrifuged at 12 000 *g* for 15 min (Velikova *et al.* 2000). An aliquot (0.5 cm^3) of the supernatant was added to 0.5 cm^3 of a phosphate buffer (pH 7.0) and 1 cm^3 of 1 M KI. The absorbance of the mixture was read at 390 nm. The H_2O_2 content was determined using the coefficient of absorbance of 0.28 μM^{-1} cm^{-1}.

The production rate of $O_2^{\bullet-}$ was measured as that described by Elstner and Heupel (1976). Fresh leaf samples (0.2 g) were homogenized in 1 cm^3 of a 50 mM phosphate buffer (pH 7.8), and the homogenate was centrifuged at 10 000 *g* for 10 min. Then, 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of a 50 mM phosphate buffer (pH 7.8) and 0.1 cm^3 of 10 mM hydroxylamine hydrochloride. After 1 h reaction at 25 °C, the mixture was added to 1 cm^3 of 17 mM sulfanilamide and 1 cm^3 of 7 mM α-naphthylamine and incubated at 25 °C for 20 min. The specific absorbance at 530 nm was determined. Sodium nitrite was used as standard solution to calculate the production rate of $O_2^{\bullet-}$.

Lipid peroxidation was determined by measuring malondialdehyde (MDA), a major thiobarbituric acid reactive species (TBARS) and product of lipid peroxidation (Heath and Packer 1968). Samples (0.2 g) were ground in 3 cm^3 of TCA (0.1 %, m/v). The homogenate was centrifuged at 10 000 *g* for 10 min and 1 cm^3 of the supernatant was mixed with 4 cm^3 of 0.5 % thiobarbituric acid in 20 % TCA. The mixture was heated at 95 °C for 30 min, chilled on ice, and then centrifuged at 10 000 *g* for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for a non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated using the coefficient of absorbance of 155 mM^{-1} cm^{-1}.

For the extraction of antioxidative enzymes, leaves and roots were homogenized with a 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.8) containing 0.2 mM EDTA and 2 % (m/v) polyvinylpyrrolidone (PVPP) using a chilled pestle and mortar. The homogenate was centrifuged at 12 000 *g* for 20 min and the resulted supernatant was used for determing enzyme activities. The whole extraction procedure was carried out at 4 °C. All spectrophotometric analyses were conducted on a *Shimadzu UV-2450* spectrophotometer (Kyoto, Japan). Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart and Bewley (1980). Catalase (CAT, EC 1.11.1.6) activity was measured as decline in absorbance at 240 nm due to the extinction of H_2O_2 according to the method of Patra *et al.* (1978). Guaiacol peroxidase (POD, EC 1.11.1.7) activity was measured based on increase in absorbance at 470 nm due to guaiacol oxidation (Nickel and Cunningham 1969).

The determination of ascorbic acid (ASA) content followed the procedure of Singh *et al.* (2006) with some modifications. Fresh tissue (1 g) was ground in liquid nitrogen, extracted with 3 cm^3 of 5 % TCA, and centrifuged at 15 000 *g* for 15 min. ASA was determined in a reaction mixture consisting of 0.2 cm^3 of the supernatant, 0.5 cm^3 of a 150 mM phosphate buffer (pH 7.4) containing 5 mM Na_2EDTA, and of 0.2 cm^3 of deionized water, and the colour developed after the addition of 0.4 cm^3 of 10 % TCA, 0.4 cm^3 of 44 % (m/m) phosphoric acid, 0.4 cm^3 of α,ά-dipyridyl in 70 % (v/v) ethanol, and 0.2 cm^3 of 3 % $FeCl_3$. The reaction mixture was incubated at 40 °C for 40 min, and the absorbance was read at 532 nm using ascorbic acid as standard.

Reduced glutathione (GSH) content was determined fluorimetrically by the method of Hissin and Hilf (1976) with some modifications. The sample was extracted in an ice bath with 3 cm^3 of a 100 mM phosphate buffer (pH 8.0) containing 5 mM Na_2EDTA, and with 1 cm^3 of 25 % (m/m) *meta*-phosphoric acid, and then centrifuged at 10 000 *g* for 30 min. The supernatant was further diluted five times with the phosphate-EDTA buffer (pH 8.0). The final assay mixture (2 cm^3) contained 0.1 cm^3 of the diluted tissue supernatant, 1.8 cm^3 of the phosphate-EDTA buffer, and 0.1 cm^3 of an *o*-phthaldialdehyde solution. After thorough mixing and incubation at room temperature for 15 min, fluorescence was recorded at 420 nm after excitation at 350 nm.

The sub-cellular distribution of Cd within leaves was determined using the protocol described by Lozano-Rodriguez *et al.* (1997) with some modifications. Leaves (25 g) were homogenized in 50 cm^3 of a chilled extraction buffer [50 mM HEPES (pH 7.5), 500 mM sucrose, 1 mM DTT, 5 mM ascorbate, and 1 % (m/v) PVPP]. The homogenate was filtered through a nylon cloth and centrifuged at 500 *g* for 5 min in order to pellet cell debris (Turner 1970). Then, the supernatant was centrifuged at 10 000 *g* for 30 min to isolate cell organelles. The supernatant was then centrifuged at 100 000 *g* for 30 min to separate the membrane-containing fraction from the soluble fraction. All the steps were performed at 4 °C. The pellets were dried and digested in 3 cm^3 of nitric acid (52 %, m/m) in *PARR* acid digestion bombs (*PARR Instrument Company*, Moline, USA). After 1 h at 150 °C, the digested samples were filtered and Cd was subsequently determined by flame atomic absorption spectroscopy. The digestion of the soluble fraction was achieved by adding 1.5 cm^3 of 52 % nitric acid to 1.5 cm^3 of the suspension (Carrier *et al.* 2003).

All data presented here are the mean values of three independent experiments with five replicates. Statistical analyses were performed by the analysis of variance (*ANOVA*) using the *SAS* software. Differences between treatments were separated by the least significant difference (LSD) test at a 0.05 probability level.

Results

The Cd treatment inhibited plant growth of both the cultivars significantly, especially of Huayu 22 (Table 1). In the absence of Cd, the addition of SA, SNP or SA+SNP had no visible effect on plant growth. However, in the Cd-treated plants, the addition of SA and SNP alleviated the Cd-inhibition on growth significantly, especially SA+SNP.

In both the cultivars, the Cd content was higher in roots than in shoots. By adding SA, SNP, or SA+SNP in the nutrient solution with Cd, the Cd accumulation in roots did not change, whereas the Cd content in shoots of both the cultivars decreased, especially by adding

SA+SNP (Table 2). The application of SA, SNP, and especially SA+SNP in the presence of Cd resulted in a significant decrease of TF in both Huayu 22 and Xiaobaisha.

Table 1. Effects of SA (0.1 mM) and SNP (0.25 mM) on plant height, root length, dry masses of shoots and roots, and leaf area of two peanut cultivars under control conditions and a Cd (200 μM) stress. Means ± SD, n = 5. Different letters in the same column indicate significant differences at $P < 0.05$.

Cultivars	Treatments	Plant height [cm]	Root length [cm]	Dry mass [mg plant^{-1}] shoots	roots	Leaf area [cm^2]
Huayu 22	controls	18.35 ± 0.87a	7.40 ± 0.18a	983.75 ± 6.65a	93.55 ± 3.32a	6.10 ± 0.05a
	SA	18.82 ± 0.84a	7.28 ± 0.42a	985.25 ± 12.04a	93.70 ± 1.76a	6.12 ± 0.03a
	SNP	18.36 ± 0.44a	7.30 ± 0.37a	988.00 ± 7.16a	94.45 ± 2.98a	6.08 ± 0.03a
	SA+SNP	19.03 ± 0.65a	7.38 ± 0.43a	987.25 ± 10.81a	97.55 ± 2.30a	6.17 ± 0.03a
	Cd	13.80 ± 0.50d	5.23 ± 0.26d	676.50 ± 10.97e	58.50 ± 3.18d	4.09 ± 0.11d
	Cd+SA	14.86 ± 0.65c	5.98 ± 0.30c	769.00 ± 14.99d	72.68 ± 2.92c	5.37 ± 0.04b
	Cd+SNP	14.93 ± 0.31c	6.08 ± 0.44bc	787.50 ± 7.00c	76.05 ± 4.17c	4.64 ± 0.28c
	Cd+SA+SNP	17.10 ± 0.59b	6.50 ± 0.29b	803.75 ± 9.74b	86.18 ± 4.02b	5.39 ± 0.09b
Xiaobaisha	controls	16.53 ± 0.71ab	6.50 ± 0.28ab	856.50 ± 26.24a	85.25 ± 3.25a	5.77 ± 0.11abc
	SA	16.43 ± 0.42ab	6.53 ± 0.43ab	874.63 ± 13.40a	85.80 ± 3.02a	5.87 ± 0.11a
	SNP	16.48 ± 0.49ab	6.48 ± 0.25ab	869.58 ± 19.70a	84.98 ± 3.67a	5.85 ± 0.09ab
	SA+SNP	16.74 ± 0.63a	6.63 ± 0.43a	873.35 ± 9.80a	86.50 ± 1.80a	5.73 ± 0.11bc
	Cd	14.08 ± 0.51d	5.10 ± 0.28e	631.73 ± 9.83d	65.00 ± 1.97d	4.90 ± 0.08e
	Cd+SA	15.20 ± 0.54c	5.63 ± 0.24cd	711.60 ± 25.89c	71.50 ± 2.41c	5.39 ± 0.08d
	Cd+SNP	15.20 ± 0.54c	5.58 ± 0.26d	708.05 ± 33.15c	72.85 ± 2.90c	5.39 ± 0.06d
	Cd+SA+SNP	15.88 ± 0.77bc	6.08 ± 0.26bc	800.73 ± 18.49b	80.75 ± 1.65b	5.71 ± 0.08c

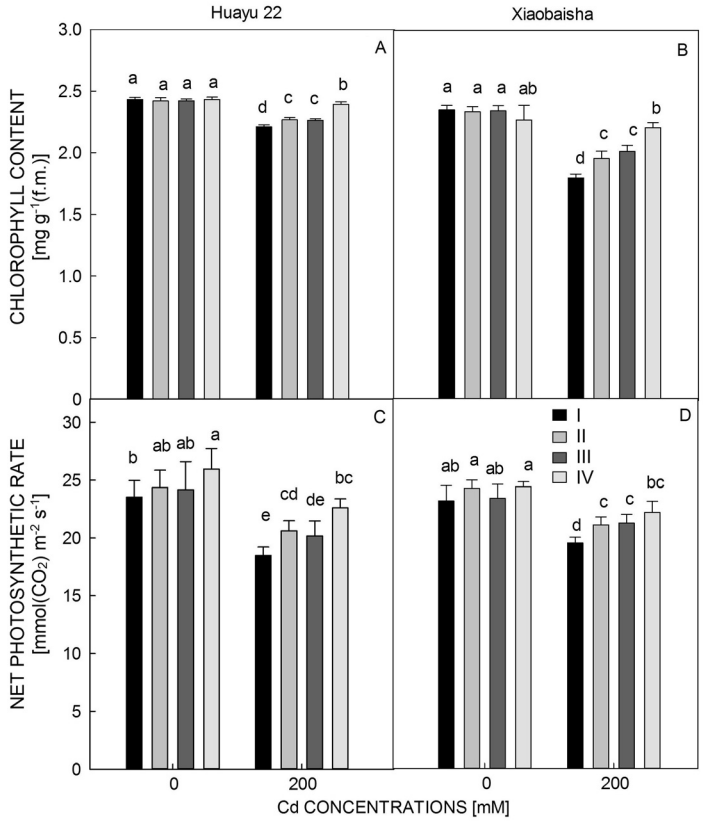

Fig. 1. Effects of Cd in combination with SA and SNP on the total chlorophyll content and net photosynthetic rate in leaves of two peanut cultivars. I - control, II - 0.1 mM SA, III - 0.25 mM SNP, IV - 0.1 mM SA + 0.25 mM SNP. Means ± SD, n = 5; different letters indicate significant differences at $P < 0.05$.

Compared with the controls, there was no significant effect of SA and SNP on Chl content of both the cultivars in the absence of Cd (Fig. 1*A*,*B*). However, an obvious degradation of Chl was detected under the Cd stress. When plants were treated with Cd in the presence of SA, SNP, and especially SA+SNP, the Chl content was significantly higher in both the cultivars compared with those treated with Cd alone.

The Cd exposure reduced the net photosynthetic rate by 21.5 and 15.8 % in Huayu 22 and Xiaobaisha, respectively, compared to the controls (Fig. 1*C*,*D*). The inhibitory effect of Cd on photosynthesis of both the cultivars was ameliorated significantly by the addition of SA or SNP, and SA+SNP had the best promoting effect.

The Cd treatment induced a dramatic increase in $O_2^{\bullet-}$ production and H_2O_2 content in leaves of both the cultivars, with a higher increase in Huayu 22 than in Xiaobaisha. The addition of SA, SNP, and particularly SA+SNP diminished the Cd-induced ROS accumulation of both the cultivars.

Table 2. Effects of SA (0.1 mM) and SNP (0.25 mM) on the Cd content [mg kg^{-1}(d.m.)] in roots and shoots and on the translocation factor (TF) of two peanut cultivars under a Cd stress (200 μM). Means ± SD, n = 5. Different letters in the same column indicate significant differences at $P < 0.05$.

Cultivars	Treatments	Cd in roots	Cd in shoots	TF
Huayu 22	Cd	728.32 ± 43.52a	169.86 ± 19.60a	23.44 ± 3.54a
	Cd+SA	727.85 ± 34.32a	106.91 ± 11.27bc	14.77 ± 2.33bc
	Cd+SNP	735.73 ± 46.29a	117.18 ± 15.24b	16.04 ± 2.88b
	Cd+SA+SNP	741.17 ± 33.70a	87.70 ± 6.76c	11.83 ± 0.52c
Xiaobaisha	Cd	744.16 ± 42.47a	112.28 ± 10.97a	15.07 ± 0.79a
	Cd+SA	733.65 ± 24.55a	89.33 ± 6.80b	12.17 ± 0.61b
	Cd+SNP	717.58 ± 19.14a	89.30 ± 6.10b	12.46 ± 1.05b
	Cd+SA+SNP	727.65 ± 31.25a	72.64 ± 6.93c	9.97 ± 0.70c

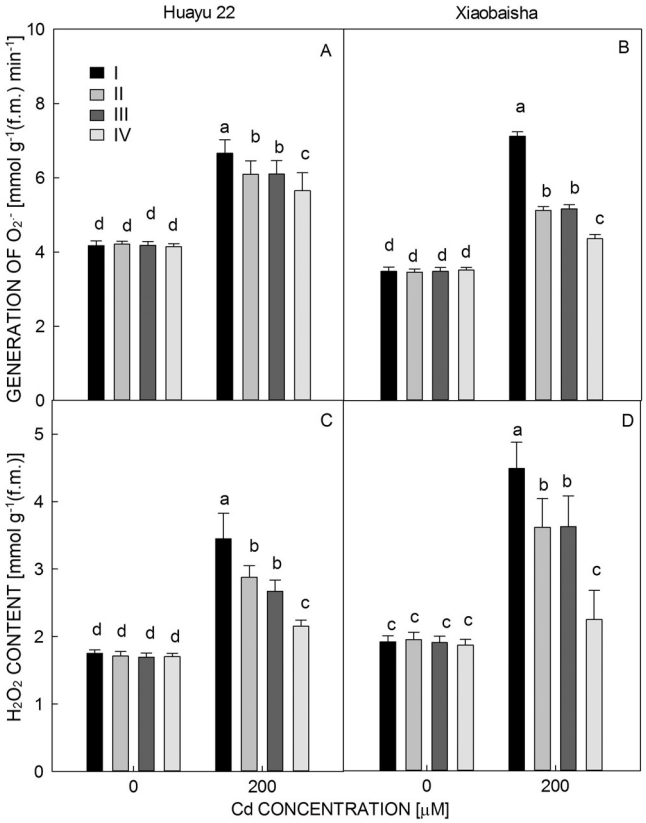

Fig. 2. Effects of Cd in combination with SA and SNP on the generation rate of $O_2^{\bullet-}$, and H_2O_2 content in leaves of two peanut cultivars. I - control, II - 0.1 mM SA, III - 0.25 mM SNP, IV - 0.1 mM SA + 0.25 mM SNP. Means ± SD, n = 5; different letters indicate significant difference at $P < 0.05$.

The MDA content is widely used as the indicator of lipid peroxidation. During the two-week experimental period, Cd alone induced a significant increase in MDA content in both the cultivars, but the increase was greater in Huayu 22. Exogenous SA or NO markedly reduced the Cd-induced MDA accumulation in both the cultivars, and the reduction was most obvious with the application of SA+SNP (Fig. 3).

The Cd exposure inhibited the SOD activity in both the cultivars (Fig. 4*A,B*) and Huayu 22 was more affected than Xiaobaisha. The addition of SA, SNP, and especially SA+SNP had a promoting effect on the Cd-inhibited SOD activity in both the cultivars. The Cd exposure caused a more significant reduction in the POD activity in Huayu 22 than in Xiaobaisha (Fig. 4 *C,D*). Compared with Cd alone, exogenous SA, NO, and SA+SNP increased the POD activity in Huayu 22. In Xiaobaisha, SA or SNP had no obvious effect on the Cd-decreased POD activity, but SA+SNP increased the POD activity significantly compared with Cd alone. After the Cd treatment, the CAT activity was inhibited dramatically in both the cultivars. The reatments with SA, SNP, and especially with SA+SNP in the presence of Cd resulted in a remarkable increase in the CAT activity of both the cultivars.

Irrespective of Cd supply, the ASA content in Xiaobaisha was higher than in Huayu 22 (Fig. 5*A,B)*. Cd alone significantly decreased the ASA content in both the cultivars, especially in Huayu 22 compared with the controls. However, exogenous SA or NO increased the

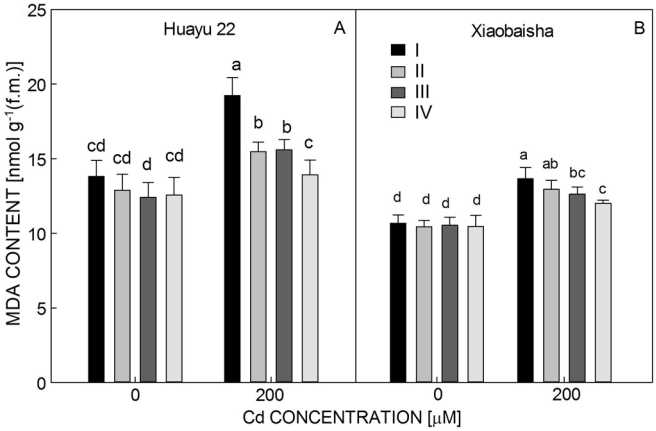

Fig. 3. Effects of Cd in combination with SA and SNP on MDA content in leaves of two peanut cultivars. I - control, II - 0.1 mM SA, III - 0.25 mM SNP, IV - 0.1 mM SA + 0.25 mM SNP. Means ± SD, n = 5; different letters indicate significant differences at $P < 0.05$.

Table 3. Effects of SA (0.1 mM) and SNP (0.25 mM) on the content of K, Ca, Mg, and Fe [mg g^{-1}(d.m.)] in leaves of two peanut cultivars under control conditions and a Cd (200 μM) stress. Means ± SD, n = 5. Different letters in the same column indicate significant differences at $P < 0.05$.

Cultivars	Treatments	K	Ca	Mg	Fe
Huayu 22	controls	50.54 ± 2.49a	3.87 ± 0.32ab	4.40 ± 0.41a	3.09 ± 0.21a
	SA	47.91 ± 3.66a	3.79 ± 0.48ab	4.54 ± 0.36a	2.81 ± 0.28abc
	SNP	48.76 ± 2.08a	3.92 ± 0.25ab	4.34 ± 0.37ab	2.92 ± 0.25ab
	SA+SNP	48.99 ± 3.34a	4.12 ± 0.25a	4.55 ± 0.40a	2.94 ± 0.25ab
	Cd	27.84 ± 1.98c	2.05 ± 0.12d	2.73 ± 0.32d	1.60 ± 0.31d
	Cd+SA	35.76 ± 2.60b	3.25 ± 0.28c	3.57 ± 0.32c	2.60 ± 0.21bc
	Cd+SNP	35.09 ± 2.45b	3.51 ± 0.36bc	3.46 ± 0.41c	2.56 ± 0.23c
	Cd+SA+SNP	47.66 ± 2.54a	3.71 ± 0.30ab	3.84 ± 0.25bc	3.04 ± 0.14a
Xiaobaisha	controls	42.76 ± 2.26a	3.86 ± 0.35ab	5.44 ± 0.56abc	3.70 ± 0.34ab
	SA	42.46 ± 2.12ab	3.37 ± 0.45bc	5.53 ± 0.30ab	3.67 ± 0.41abc
	SNP	43.76 ± 3.21a	3.47 ± 0.50abc	5.47 ± 0.57ab	3.83 ± 0.35a
	SA+ SNP	42.37 ± 3.02ab	3.91 ± 0.26a	5.71 ± 0.39a	3.80 ± 0.33a
	Cd	26.02 ± 1.92d	2.09 ± 0.14d	3.25 ± 0.34d	2.30 ± 0.21d
	Cd+SA	36.41 ± 2.31c	3.03 ± 0.36c	4.64 ± 0.45c	3.21 ± 0.09c
	Cd+SNP	39.06 ± 2.03bc	3.09 ± 0.08c	4.89 ± 0.62bc	3.24 ± 0.34bc
	Cd+SA+SNP	40.70 ± 1.21ab	3.40 ± 0.36bc	5.67 ± 0.44ab	3.66 ± 0.38abc

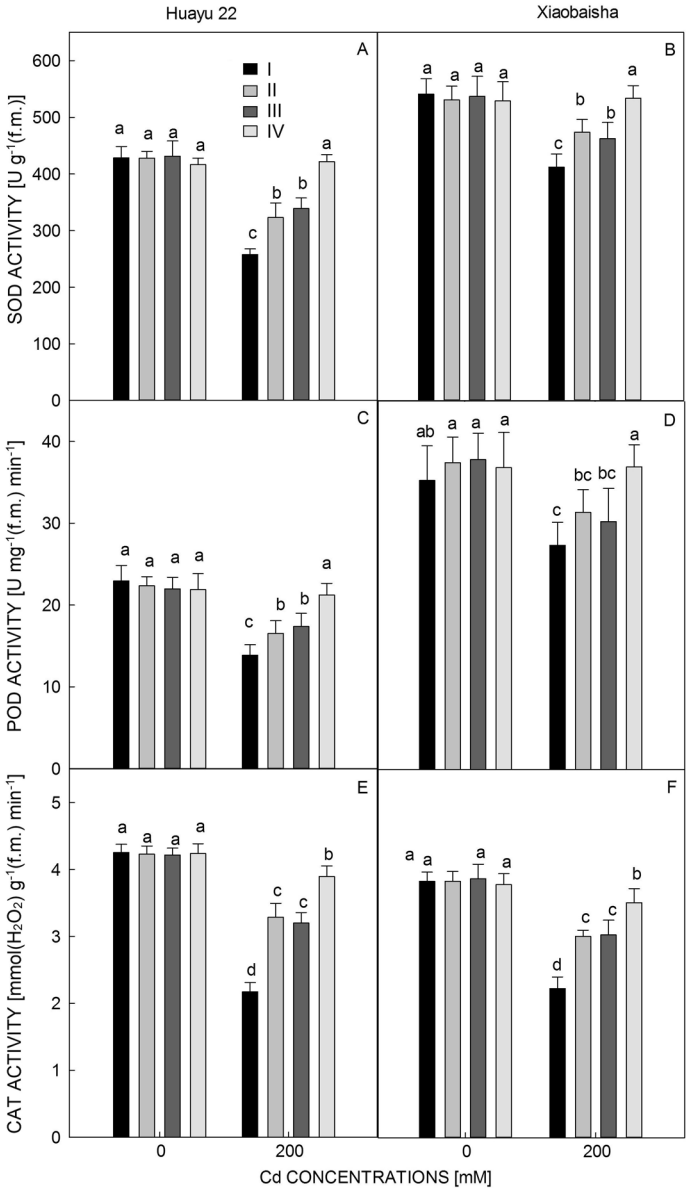

Fig. 4. Effects of Cd in combination with SA and SNP on the activities of SOD (*A,B*), POD (*C,D*), and CAT (*E,F*) in leaves of two peanut cultivars. I - control, II - 0.1 mM SA, III - 0.25 mM SNP, IV - 0.1 mM SA + 0.25 mM SNP. Means ± SD, $n = 5$; different letters indicate significant differences at $P < 0.05$. One unit of SOD is defined as the amount of enzyme required to cause a 50 % inhibition of the NBT photoreduction rate. One unit of POD is quantified by the amount of tetraguaiacol formed using its coefficient of absorbance of 26.6 mM^{-1} cm^{-1}.

Cd-decreased content of ASA in both the cultivars, especially SA+SNP in Huayu 22. Both the cultivars treated with Cd showed a significant decrease in the GSH content compared with the controls (Fig. 5*C,D)*. The reduction in Huayu 22 was more significant than in Xiaobaisha. The inhibitory effect of Cd on the GSH content was significantly ameliorated in the presence of SA, SNP, and especially SA+SNP.

In the Cd-treated plants, the K, Ca, Mg, and Fe content significantly decreased in both the cultivars compared with the controls. The applications of SA, SNP, and especially SA+SNP increased the content of these nutrients in both the cultivars (Table 3).

In root tissue of the Cd-stressed plants, most Cd was in the cell wall in Huayu 22, whereas most Cd accumulated in soluble cytoplasmic fractions in Xiaobaisha. The least Cd was located in cell organelles of both the cultivars. In leaf tissues of both the cultivars, most Cd was in the cell wall, and a minority of Cd accumulated in cell organelles. In roots and leaves of both the cultivars, the application of SA+SNP increased the Cd content in the cell wall and decreased the Cd accumulation in the soluble fractions and cell organelles.

Table 4. Effects of SA (0.1 mM) and SNP (0.25 mM) on the Cd subcellular distribution in roots and leaves of two peanut cultivars under a Cd (200 μM) stress. Means ± SD, $n = 5$. Different letters in the same column indicate significant differences at $P < 0.05$.

Cultivars	Treatments	Cd in roots [μg g^{-1}(f.m.)]			Cd in leaves [μg g^{-1}(f.m.)]		
		cell wall	soluble fraction	cell organelle	cell wall	soluble fraction	cell organelle
Huayu 22	Cd	97.93 ± 4.76d	69.90 ± 5.50a	46.33 ± 4.18a	9.76 ± 0.58c	3.90 ± 0.22a	1.91 ± 0.12a
	Cd+SA	119.88 ± 5.91c	58.03 ± 3.57b	32.11 ± 2.33b	13.23 ± 0.64b	2.29 ± 0.25bc	1.39 ± 0.09b
	Cd+SNP	134.16 ± 8.28b	52.73 ± 3.05b	29.08 ± 2.11b	13.34 ± 0.56b	2.59 ± 0.32b	1.34 ± 0.06b
	Cd+SA+SNP	44.97 ± 2.69c	153.32 ± 7.46a	23.76 ± 1.98c	15.14 ± 0.25a	1.97 ± 0.08c	0.97 ± 0.07c
Xiaobaisha	Cd	44.30 ± 2.15d	149.30 ± 2.78a	43.07 ± 1.97a	8.31 ± 0.32c	3.06 ± 0.23a	1.25 ± 0.10a
	Cd+SA	57.72 ± 3.48c	134.36 ± 4.94b	32.95 ± 2.39b	10.69 ± 0.43b	2.43 ± 0.29b	0.98 ± 0.05b
	Cd+SNP	62.56 ± 2.83b	125.99 ± 6.14c	30.26 ± 1.42b	11.11 ± 0.39b	2.14 ± 0.19b	0.94 ± 0.07b
	Cd+SA+SNP	67.12 ± 3.00a	102.68 ± 5.19d	24.84 ± 2.10c	13.99 ± 0.85a	1.52 ± 0.20c	0.78 ± 0.05c

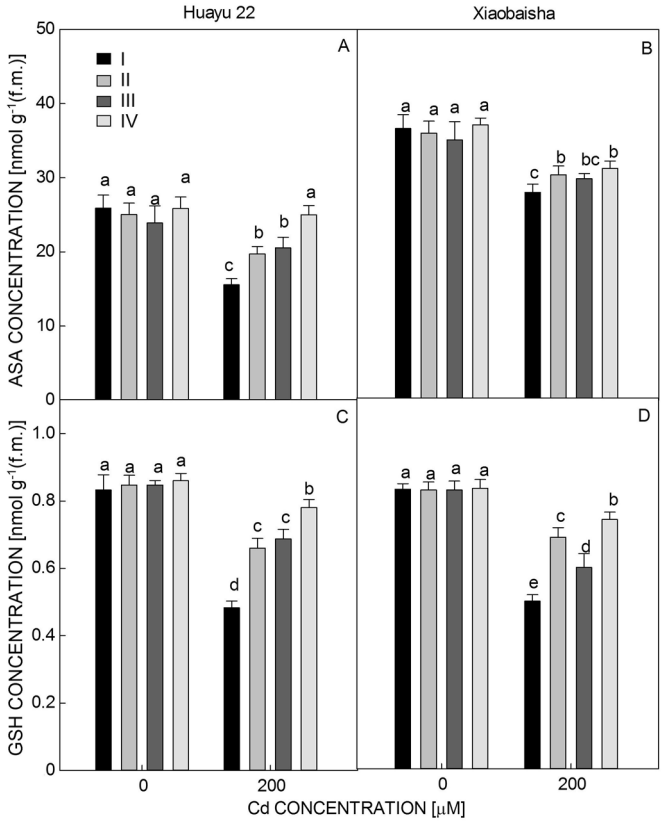

Fig. 5. Effects of Cd in combination with SA and SNP on the content of ASA and GSH in leaves of two peanut cultivars. I - control, II - 0.1 mM SA, III - 0.25 mM SNP, IV - 0.1 mM SA + 0.25 mM SNP. Means ± SD, $n = 5$; different letters indicate significant differences at $P < 0.05$.

Discussion

SA and NO have been reported to induce a number of defence responses to abiotic stresses (Shi *et al.* 2009, Kazemi *et al.* 2010, Wang *et al.* 2013b). In this work, we analyzed the possible roles of exogenous SA and SNP (a donor of NO) in the amelioration of Cd toxicity in two peanut cultivars (Huayu 22 and Xiaobaisha). Cd alone induced pronounced growth retardation in both the cultivars and especially in Huayu 22 (Table 1). This indicates that Huayu 22 was more sensitive to the Cd stress. The addition of SA and SNP to the medium with Cd effectively alleviated the Cd-stress. It has been shown that SA and NO act as plant growth regulators that can separately or together counteract a Cd-induced decrease in plant growth (Shi *et al.* 2009, Xu *et al.* 2010, Wang

et al. 2013b). Many studies reported that SA reduces detrimental effects of Cd on different plants (Guo *et al.* 2007, 2009). Also, the alleviation of Cd toxicity by NO was reported (Chen *et al.* 2010, Wang *et al.* 2013a). In our experiments, the combination of SA and SNP strikingly enhanced peanut growth under the Cd stress. Our previous study also revealed that the combination of SA and SNP alleviate a Cd stress on perennial ryegrass significantly (Wang *et al.* 2013b).

Plant roots touch Cd ions in soil directly. Therefore, the Cd content was higher in roots than in shoots of both the cultivars when they were exposed to Cd (Table 2). Plant roots are the main site of Cd immobilization, mainly by means of the retention in cell walls (Wagner 1993) and building up some efficient barriers restricting Cd entry into the xylem, and thus preventing its translocation into shoots (Vaculík *et al.* 2009, Lux *et al.* 2011). Both peanut cultivars studied in our experiments might be considered as "Cd-shoot excluder" with Cd accumulated much more in roots than in shoots. This behaviour is one of the several strategies of tolerance to Cd (Lozano-Rodriguez *et al.* 1997). The results from this study demonstrate that the Cd content in leaves of the peanut seedlings under the Cd stress was cultivar-dependent, and the Cd accumulation in leaves was lower in Xiaobaisha than in Huayu 22. This indicates that Xiaobaisha had a stronger ability to inhibit Cd transferred to shoots. Further, the addition of SA or SNP decreased the Cd accumulation in shoots of both the cultivars, although the Cd content in roots was not affected (Table 2). The lower Cd content in SA- or SNP-exposed shoots was caused by a decreased Cd translocation but not by a reduced Cd uptake. Therefore, it is proposed that SA or SNP decreased the root-to-shoot translocation of Cd resulting in the low Cd accumulation in shoots. A decreased Cd accumulation in shoots mediated by SA was found in maize (Krantev *et al.* 2008) and hemp (Shi *et al.* 2009). Moreover, an NO-induced decrease of Cd translocation was reported in many plants (Xiong *et al.* 2009, Wang *et al.* 2013a). Our previous study also revealed that the application of SA and SNP decreases Cd translocation from roots to shoots (Wang *et al.* 2013b). The reason of a minority of Cd in peanut shoots might be that SA and NO protected the plants from the membrane damage. The low Cd accumulation in shoots may be an important feature of SA- or NO-increased Cd tolerance.

Decreasing leaf Chl content is one of the most general effects of Cd on plants (Chen *et al.* 2008). A more notable reduction of Chl content was detected in Huayu 22 than Xiaobaisha. The Cd-induced decrease in the Chl content was partially reversed when the two cultivars were treated with SA, SNP, and especially SA+SNP (Fig. 1). Shi *et al.* (2009) demonstrated that a treatment with SA promotes protection of the photosynthetic pigments under a Cd stress. Moreover, the increase of Chl synthesis induced by the application of SNP was reported by Chen *et al.* (2010). The Cd-induced decrease of the Chl content was accompanied by the reduction of the net photosynthetic rate. Generally, Cd-causes not only a decrease in chlorophyll content, but also a destruction of chloroplast ultrastructure, a decrease in photochemistry, carboxylating enzyme activities, and lipid peroxidation (Krantev *et al.* 2008). Exogenous SA and NO increased photosynthesis of both the peanut cultivars, and the alleviating effect of SA+SNP was most obvious, which is important for improving Cd tolerance of peanut seedlings.

In both the peanut cultivars, the Cd exposure led to the accumulation of $O_2^{\bullet-}$ and H_2O_2. Moreover, the Cd-caused overaccumulation of $O_2^{\bullet-}$ and H_2O_2 in Huayu 22 was more serious than in Xiaobaisha, so it might be concluded that Huayu 22 is a more sensitive cultivar to Cd stress. However, Cd-induced $O_2^{\bullet-}$ and H_2O_2 in leaves of the peanut seedlings was markedly eliminated by exogenous SA or NO, especially SA+SNP for Huayu 22 (Fig. 2). In the case of metal toxicity, especially Cd, plants have developed detoxification mechanisms related with some stress signal molecules, such as SA and NO (Rodríguez-Serrano *et al.* 2006). SA acts as potential non-enzymatic antioxidant for eliminating ROS injury (Kang *et al.* 2013). NO can indirectly decrease ROS accumulation by elevating SOD, APX, and CAT activities (Chen *et al.* 2010, Wang *et al.* 2013a). Several reports demonstrated that the combined application of SA and SNP effectively inhibits ROS damage (Kazemi *et al.* 2010, Simaei *et al.* 2011, Wang *et al.* 2013b).

The increased MDA content is an index of the loss of membrane integrity and lipid peroxidation. The results show that Cd caused an MDA accumulation in both the cultivars, especially in Huayu 22 (Fig. 3). The increase in $O_2^{\bullet-}$ and H_2O_2 under Cd stress probably accounts for the excess accumulation of MDA (Simaei *et al.* 2011). It is interesting that the Cd-induced MDA accumulation decreased in the SA- and NO-treated plants, and the alleviated effect was more obvious in Huayu 22 with the addition of SA+SNP (Fig. 3). SA participates in the stabilization of cell membranes (Mishra and Choudhuri 1999) and NO increases the antioxidative ability (Chen *et al.* 2010, Wang *et al.* 2013a), which may be responsible for the lowered MDA content.

Numerous investigations have indicated that the up-regulation of the antioxidant systems is an early response of plants to environmental stress (Vital *et al.* 2008). However, the Cd stress inhibited the activities of SOD, POD, and CAT in both the cultivars, and the inhibition was more serious in Huayu 22 (Fig. 4). It might be speculated that a better antioxidative ability occurred in Xiaobaisha than in Huayu 22. It is worth noting that exogenous SA increased the activities of SOD, POD, and CAT in the Cd-stressed plants of both the cultivars (Fig. 4). Similarly, in the case of maize (Krantev *et al.* 2008), rice (Guo *et al.* 2009), and wheat (Kang *et al.* 2013), the increased antioxidant activity occurs in SA-pretreated plants. Moreover, exogenous NO also

enhanced the activities of antioxidant enzymes (SOD, POD and CAT) in this study. The enhancement of antioxidant system by exogenous NO was also demonstrated in barley, *Medicago truncatula* seedlings, and perennial ryegrass (Chen *et al.* 2010, Xu *et al.* 2010, Wang *et al.* 2013a). In addition, SA and NO synergistically promoted the activities of antioxidant enzymes in the peanut seedlings (Fig. 4). Lamattina *et al.* (2003) reported that NO might regulate the expression of respective genes. The synergistic effect of SA and NO was also reported under nickel stress (Kazemi *et al.* 2010) and under salinity (Simaei *et al.* 2011).

ASA and GSH are scavengers of ROS preventing or at least alleviating deleterious effects caused by ROS, thus providing membrane protection (Ahmad *et al.* 2009). Cd decreased the content of ASA and GSH in both the cultivars significantly, especially in Huayu 22, and the treatments with SA and SNP increased the ASA and GSH content (Fig. 5). ASA and GSH can participate in detoxifying ROS directly or through certain enzymes restricting lipid peroxidation and oxidative stress. This mechanism may play an important role in increasing plant resistance to oxidative damage.

Until now, a few experiments showed that exogenous SA and NO influence nutrient absorption under Cd stress (Drazic *et al.* 2006, Xu *et al.* 2010). Accumulated Cd in plants seems to compete for the same transmembrane carriers as nutrients, such as K, Ca, Mg, Fe, Cu, and Zn (Sanita di Toppi and Gabbrielli 1999), which might be responsible for Cd-induced decrease in K, Ca, Mg, and Fe content. Exogenous SA and NO reduced the Cd uptake but increased the content of K, Ca, Mg, and Fe. In addition, H^+-ATPase in the plasma membrane plays an important role in the transport of multiple ions (Palmgren and Harper 1999), and there are investigations indicating that SA and NO could increase H^+-ATPase activity (Wang *et al*. 2013a), which may also account for SA and SNP induced absorption of K, Ca, Mg, and Fe. The effect of SA on mineral uptake under Cd stress was reported by Drazic *et al.* (2006). Furthermore, there are also studies revealing that NO promotes Cd-affected ion uptake (Xu *et al.* 2010, Wang *et al.* 2013a). Our previous study demonstrated that SA+SNP promotes a better absorption of nutrients under Cd stress than the application of SA or SNP individually (Wang *et al.* 2013b).

In the present work, we found a different Cd subcellular distribution in roots of both the cultivars studied. In Huayu 22, most Cd accumulated in the cell wall, whereas a majority of Cd was in the soluble fractions of Xiaobaisha. Similarly, the lowest Cd content was in cell organelles of both the cultivars (Table 3). Cd retention in the cell wall might be due to cross-linking Cd to carboxyl groups of the cell wall (Barceló and Poschenrieder 1990) and/or to an interaction with thiol residues of soluble proteins (Leita *et al.* 1993). Cd compartmentalization in vacuoles plays a main role in storing excess Cd (Xiong *et al.* 2009). Ma *et al.* (2005) found that Cd is mostly accumulated in the cell wall and soluble fractions. In leaves, the Cd content in the cell wall of both the cultivars was largest, and the Cd accumulation in organelles was lowest. The least Cd accumulation in organelles of roots and leaves might be a strategy of the peanut plants to increase Cd tolerance.

An important finding from this study is that SA and NO increased a Cd accumulation in the cell wall and decreased a Cd distribution to cell organelles of both the cultivars (Table 3), which may play an important role in the improvement of Cd tolerance. There are only few reports on the effects of SA on Cd subcellular distribution. According to our views, the possible mechanism of SA might be thickening Casparian strips in the endodermis as well as thicker cell walls of xylem and pericycle (Da Cunha and do Nascimento 2009), which might account for most Cd accumulated in the cell wall. Xiong *et al.* (2009) indicated that an SNP-induced increase of Cd accumulation in the cell wall is dependent on an SNP-induced increase of content of pectins and hemicelluloses (Xiong *et al.* 2009).

In summary, the Cd exposure alone depressed plant growth and caused Cd stress in both the cultivars, but more in Huayu 22 than in Xiaobaisha. Thereby, Xiaobaisha is regarded as more Cd-tolerant than Huayu 22. SA and NO played important roles in alleviating Cd toxicity in the peanut seedlings. Interactive effects of SA+SNP were more effective compared to the separate applications of SA or SNP.

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