

Nitric Oxide Increases Pb Tolerance by Lowering Pb Uptake and Translocation as well as Phytohormonal Changes in Cowpea (*Vigna unguiculata* (L.) Walp.)

(Nitrik Oksida Meningkatkan Toleransi Pb dengan Menurunkan pengambilan Pb dan Translokasi serta Perubahan Fitohormon dalam Kacang Panjang (*Vigna unguiculata* (L.) Walp.))

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ABSTRACT

Lead (Pb) is one of the most abundant toxic heavy metals which adversely affected growth and yield of crop plants. Nitric oxide (NO), an endogenous signaling molecule, has been suggested to be involved in defense responses to biotic and abiotic stresses in plants. The present study was done to induce Pb tolerance in cowpea plants by exogenous NO application using two levels of Pb, 0 and 200 mg Pb (NO₃)₂ kg⁻¹ soil and three NO levels, 0, 0.5 and 1 mM sodium nitroprusside (SNP), as NO donor. The results showed that Pb treatment caused a significant increase in Pb concentration in all plant parts. Roots had higher levels of Pb than the stems, leaves and seeds. Furthermore, lead toxicity reduced auxin (IAA), cytokinin and gibberellic acid (GA₃) content but increased abscisic acid (ABA) level. Moreover Pb stress decreased stomatal conductance, leaf area and consequently seed yield of cowpea. Exogenous application of NO at 0.5 mM noticeably alleviated the lead toxicity by improving the leaf area, stomatal conductance and seed yield. NO increased Pb tolerance by lowering Pb uptake and translocation, enhancing the promoting phytohormone (IAA, cytokinin and GA₃) level and reducing ABA content.

Keywords: Leaf area; Pb toxicity; plant hormones; seed yield; stomatal conductance

ABSTRAK

Plumbum (Pb) merupakan salah satu logam berat toksik paling banyak yang telah menjejaskan pertumbuhan dan hasil tanaman tumbuhan. Nitrik oksida (NO), molekul isyarat endogen disyaki terlibat dalam tindakan pertahanan terhadap stres biotik dan abiotik dalam tumbuh-tumbuhan. Kajian ini dijalankan untuk mengaruh toleransi Pb dalam tumbuhan kacang panjang dengan aplikasi NO eksogen menggunakan dua tahap Pb, 0 dan 200 mg Pb (NO₃)₂ kg⁻¹ tanah dan tiga peringkat NO, 0, 0.5 dan 1 mM sodium nitroprusid (SNP), sebagai pemberma NO. Keputusan kajian menunjukkan bahawa rawatan Pb telah menyebabkan peningkatan ketara dalam kepekatan Pb pada semua bahagian tumbuhan. Akar mempunyai tahap Pb yang lebih tinggi daripada batang, daun dan biji benih. Selain itu, ketoksikan plumbum mengurangkan kandungan auksin (IAA), sitokinin dan asid giberelik (GA₃) tetapi meningkatkan tahap asid absisik (ABA). Tambahan pula tekanan Pb mengurangkan konduktans stoma, keluasan daun dan penghasilan biji benih kacang panjang. Aplikasi eksogen NO pada 0.5 mM didapati mengurangkan keracunan plumbum dengan memperbaiki keluasan daun, konduktans stoma dan hasil biji benih. NO meningkatkan toleransi Pb dengan mengurangkan penyerapan Pb dan translokasi, menggalakkan peningkatan tahap fitohormon (IAA, sitokinin dan GA₃) serta mengurangkan kandungan ABA.

Kata kunci: Hasil benih; hormon tumbuhan; keluasan daun; ketoksikan PB; konduktans stoma

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) being a warm climate and drought tolerant crop is grown throughout the tropics particularly in the semi-arid and low rainfall regions. It provides food, fodder and also improves the soil fertility. It contains 25% protein and 64% carbohydrate. Cowpea form the major source of plant protein, vitamins to man and feed to animals and it is often called the poor man's meat. It is important mainly because it has high protein content and it adapts to different types of soil and it is also important in food security, trade and therefore in poverty reduction (Ayanwuyi & Akintonde 2012).

Contamination of agricultural soil by heavy metals has become a critical environmental concern due to their

potential adverse ecological effects. Such toxic elements are considered as soil pollutants due to their widespread occurrence and their acute and chronic toxic effect on plants grown of such soils (Nagajyoti et al. 2010). Lead (Pb) has gained considerable attention as a potent heavy metal pollutant due to the growing anthropogenic pressure on the environment (Sharma & Dubey 2005). Besides natural weathering processes, the main sources of Pb pollution are exhaust fumes of automobiles, chimneys of Pb factories using Pb, effluents from the storage battery, industry, mining and smelting of Pb ores, metal plating and finishing operations, fertilizers, pesticides and additives in pigments and gasoline (Eick et al. 1999). Pb contaminated soils show a sharp decline in crop productivity. Although

not an essential element for plants, lead is taken up mainly through the root system and partly in minor amounts through the leaves. After entering the cell, Pb inhibits activities of many enzymes, upsets mineral nutrition and water balance, changes the hormonal status and affects membrane structure and its permeability. Visual non-specific symptoms of Pb toxicity are stunted growth, chlorosis and blackening of the root system. Pb decreases photosynthetic rate by distorting chloroplast ultrastructure, diminishing chlorophyll synthesis, obstructing electron transport and inhibiting activities of Calvin cycle enzymes (Sharma & Dubey 2005). Lead toxicity also causes inhibition of ATP production, lipid peroxidation and DNA damage by over production of reactive oxygen species (Pourrut et al. 2011).

Nitric oxide (NO) has emerged as an important signaling molecule associated with many biochemical and physiological processes in plants. NO is classified as a phytohormone that might function as a gaseous endogenous plant growth regulator as well as a nontraditional plant growth regulator. It has the capability to regulate diverse physiological processes in a concentration-dependent manner, such as root organogenesis, hypocotyl growth, defense responses, stomatal movement, apoptosis, hypersensitive responses, growth and development and phytoalexin production under different environmental conditions (Hayat et al. 2010). NO has been suggested to be involved in defense responses to biotic and abiotic stresses. It has been reported to exert a protective effect in response to drought stress (Shehab et al. 2010), osmotic stress (Tan et al. 2008), salt stress (Habib et al. 2013; Kausar et al. 2013), chilling stress (Liu et al. 2011), heat stress (Hasanuzzaman et al. 2012), ultraviolet-B radiation (An et al. 2005) and heavy metals stress (Esim & Atici 2013; Jhanji et al. 2012; Kumari et al. 2010; Singh et al. 2009; Wang & Yang 2005). Nonetheless there is little information on the effect of NO application on lead toxicity tolerance in plants. The objective of this study therefore was to investigate the influences of Pb stress and exogenous NO on phytohormones and Pb content changes in cowpea plants.

MATERIALS AND METHODS

PLANT MATERIAL AND EXPERIMENTAL DESIGN

In order to investigate the effect of lead stress and exogenously NO on phytohormones and Pb content changes in cowpea plants, a pot experiment was done at summer 2014 in research field of the Yadegar-e-Imam Khomeini (RAH) Shahre-rey Branch, Islamic Azad University,

Tehran, Iran (51°28'E, 35°35'N; 1000 m). Cowpea (*Vigna unguiculata* cv. Kamran) seeds without visible defect, insect damage and malformation were surface sterilized using 5% sodium hypochlorite solution for 5 min and then rinsed 3 times with sterile distilled water. After sterilization, the seeds were soaked in different levels (0, 0.5 and 1 mM) of sodium nitroprusside (SNP) as NO donor for 20 h. The seeds were then sown in 50 cm in diameter and depth plastic pots filled with 20 kg soil containing an equal mixture of peat, decomposed manure and farm soil. Soil characteristics of the pots are presented in Table 1. Before sowing, the soil of pots was mixed with appropriate amount of Pb (NO₃)₂ to supply 0 and 200 mg kg⁻¹ soil. The experiment was done in a completely randomized design with three replicates and four treatments: control, Pb stress, Pb + 0.5 mM SNP and Pb + 1 mM SNP. The sowing was done on 20th June 2014 and then pots were placed in farm conditions. In each pot 20 seeds were sown in 3 cm depth. After thinning at 3-leafy stage, 6 seedlings remained. Irrigation was carried out regularly at the plant needs using tap water.

ASSAY OF PLANT HORMONES

Plant hormone levels were measured at the flowering stage. For estimation of growth regulators, 3-5 g fresh samples of the youngest fully expanded leaf were frozen in liquid nitrogen and stored at -80°C until analysis. The method of extraction in ethanol and the fraction of the ethanol extract were carried out according to the method described by Shindy and Smith (1975). The acidic fraction contained the acidic hormones (IAA, GA₃ and ABA) while the aqueous fraction comprised the cytokinin. Phytohormones were analyzed using high performance liquid chromatography (Agilent 1200 HPLC system, Agilent Technologies Inc., USA). The samples were resolved on a reversed phase C18 column (Eclipse XDB C18, 4.6×150 mm) with a diode array detector. Binary solvent gradient elution (A 50% methanol; B 50% methanol and 1.2% acetic acid) was used as follows - initial condition 50% B, 3 min 50%, 7 min 60%, 8 min 50% and 10 min 50%. Cytokinin, GA₃ and IAA were detected at the wavelength 254 nm, while ABA at 280 nm. IAA, Z, GA₃ (Duchefa, Netherlands) and ABA (Alfa Aesar GmbH, Germany) standards for identification and quantification of phytohormones were used.

MEASUREMENT OF STOMATAL CONDUCTANCE AND LEAF AREA

Stomatal conductance was measured on sunny days between 10:00 and 11:00 hours on the youngest fully expanded leaves using a Portable Leaf Porometer SC-1,

TABLE 1. Soil characteristics of pots

EC (dS m ⁻¹)	pH	OC (%)	N (%)	P (ppm)	K (ppm)	Pb (ppm)	Texture
1.6	7.1	3.2	0.31	20.2	475	1.4	Sandy loam

Decagon Devices, USA. Leaf area was also calculated using Leaf Area Meter CI-202, CID, Bio-Science, USA.

ASSAY OF LEAD CONTENT IN DIFFERENT PLANT PARTS

At the physiological maturity stage, the harvested cowpea plants were washed thoroughly with tap water, distilled water and deionized water in sequence. Plant samples were then separated into root, stem, leaf and seed, dried at 80°C in an oven for 48 h and were ground into powder. Each sample (0.5 g) was dry-ashed, extracted with HCl and centrifuged at 3600 rpm for 15 min. Concentrations of Pb in root, stem, leaf and seed were determined by flame atomic absorption spectrometry (Bharwana et al. 2014).

STATISTICAL ANALYSIS

Finally, statistical analysis was carried out by one-way ANOVA using MSTAT-C statistical software and the means were compared by Tukey's test at the 5% probability level.

RESULTS AND DISCUSSION

PHYTOHORMONAL CHANGES

When cowpea plants were raised under increasing concentration of lead, a decrease in the content of IAA, cytokinin and GA₃ was observed but ABA level was increased. Our study showed that Pb stress markedly decreased growth promoting hormones (IAA, cytokinin and GA₃) level in cowpea plants at 45, 38 and 33%, respectively, as compared to control, while reversibly increased ABA content at 54%. Nonetheless, under lead stress, SNP pretreatment especially at 0.5 mM elevated promoting hormones (IAA, cytokinin and GA₃) level but decreased ABA content in cowpea plants as compared to Pb treatment alone. 0.5 and 1 mM concentrations of SNP raised IAA level by 31 and 11%, cytokinin level by 24 and 9% and GA₃ level by 18 and 7% respectively, compared to the Pb treatment alone. At two concentrations of SNP, ABA level was decreased by 24 and 17% respectively, as

compared to Pb treatment alone (Figure 1). The primary cause of cell growth inhibition arises from a lead-induced simulation of IAA oxidation (Nagajyoti et al. 2010). Veselov et al. (2003) showed that a sharp reduction in cytokinin content was observed within 2 hours of supplying cadmium in wheat seedlings. The reduction in cytokinin content was probably as a result of hormone breakdown. In support of this, Cd-treatment elevated cytokinin oxidase activity. Yakhin et al. (2009) also observed that the effect of cadmium acetate was manifested in a considerable decrease in the content of phytohormones-cytokinins (isopentenyl adenine (iP), isopentenyl adenosine (iPA), zeatin, zeatin riboside, dehydrozeatin and dehydrozeatin riboside), IAA and ABA both in roots and sprouts of wheat seedlings. On the other hand, many researchers reported an increase in the level of ABA under exposure to heavy metals such as lead (Atici et al. 2005; Cenkci et al. 2010). In the present study, exogenous NO elevated IAA, cytokinin and GA₃ content but decreased ABA level under lead stress. NO induced the elongation of maize root segments in a dose-dependent manner (Gouvea et al. 1997). It has, therefore, been proposed that the IAA and NO might share some common steps in the signal transduction pathway because both elicit the same responses in plants. The dependence of IAA on NO in the induction of adventitious root development was recently demonstrated in cucumber explants (Pagnussat et al. 2002). NO has also been reported to slightly increase the chlorophyll level in wheat seedlings grown in the dark (Beligni & Lamattina 2000). Thus, the effect of NO is similar to that of cytokinin. As mentioned earlier, cytokinin regulates the synthesis of some pigments such as anthocyanins and betacyanins. NO plays the same role as cytokinin action on betacyanin accumulation (Hayat et al. 2010). The germination of lettuce seed (cv. Grand Rapid) is also a phytochrome-dependent process, and it was observed that NO donors were able to stimulate germination in the dark, similar to GA₃, or under a few minute pulse of white light. However, seeds were also able to germinate in light in the presence of NO scavenger, suggesting that light and NO can stimulate germination in different ways (Beligni & Lamattina 2000).

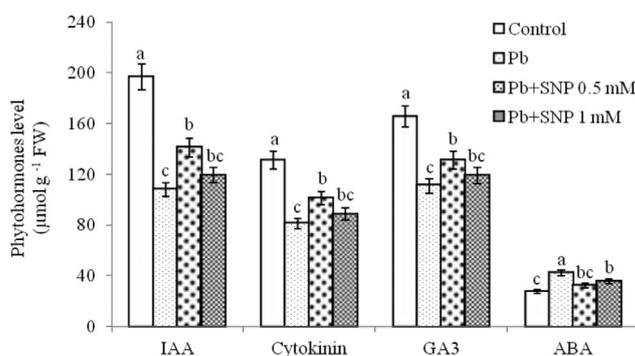


FIGURE 1. Effect of Pb (200 mg kg⁻¹ soil) and SNP (0.5 and 1 mM as NO donor) on phytohormone level of cowpea. Different letters in each phytohormone indicate significant differences at $p \leq 0.05$ level using Tukey's test. Error bars show SE

LEAD CONTENT IN DIFFERENT PLANT PARTS

The Pb treatment caused a significant increase in Pb concentration in all four plant parts (Figure 2). An increase by 12.7, 16.9, 15.4 and 15-folds in Pb content comparing to control plants was found in the roots, stems, leaves and seeds, respectively, under Pb stress. 50% of absorbed Pb was retained in the roots. In Pb treated soil, Pb concentration in the roots was 58%, 3.4 and 11.9-folds more than its concentrations in the stems, leaves and seeds, respectively. Nevertheless, under lead stress, SNP application decreased Pb content in all the plant parts. 0.5 and 1 mM concentrations of SNP declined total Pb in cowpea plants by 50 and 26% respectively, compared with the Pb treatment alone. These results indicated that NO might ameliorate the toxic effects of Pb by reducing Pb uptake and translocation in cowpea plants. It was reported that plants have developed various tolerance and resistance mechanisms in order to diminish the heavy metal stress. One of these mechanisms is to hold the heavy metal in the root and prevent the distribution to the leaves (Fernandes & Henriques 1991). Based on lead accumulation in roots more than other parts of the plant, it could be concluded that roots play a very significant role in extra lead storage. It has been shown that lead can adhere to roots cell wall, especially in pyrophosphate form (Marschner 1995). Once lead has penetrated into the root system, it may accumulate there or may be translocated to aerial plant parts. For most plant species, the majority of absorbed lead (approximately 95% or more) is accumulated in the roots and only a small fraction is translocated to aerial plant parts. When entering the root, lead mainly moves by apoplast and follows water streams until it reaches the endodermis (Lane & Martin 1977; Tanton & Crowdy 1971). There are several reasons why the transport of lead from roots to aerial plant parts is limited. These reasons include immobilization by negatively charged pectins within the cell wall, precipitation of insoluble lead salts in intercellular spaces (Islam et al. 2007; Kopittke et al. 2007), accumulation in plasma membranes (Islam et al. 2007; Jiang & Liu 2010; Seregin et al. 2004), or sequestration in the vacuoles of rhizodermal and cortical cells (Kopittke et al. 2007; Seregin et al. 2004). However, these reasons are not sufficient to explain the low

rate of lead translocation from root to shoot. The endoderm, which acts as a physical barrier, plays an important role in this phenomenon. Indeed, following apoplastic transport, lead is blocked in the endodermis by the casparian strip and must follow symplastic transport. In endodermis cells, the major part of lead is sequestered or excreted by plant detoxification systems (Poutrut et al. 2011). Transportation of metals from plant roots to shoots requires movement through the xylem (Verbruggen et al. 2009) and, when it occurs, is probably driven by transpiration (Liao et al. 2006). Although in the present study, under lead stress conditions, just 50% of the total absorbed Pb by cowpea plants was maintained in the roots which indicates cowpea was sensitive to Pb but exogenous NO not only decreased total Pb uptake but also lowered translocation of Pb to aerial parts of plants. Similar to our results, Bharwana et al. (2014) reported that Pb addition caused a significant increase in Pb concentration in all three observed plant parts of cotton, with the roots having significantly higher levels than the stems and leaves. On the other hand, Kumari et al. (2010) also found that different parts of chickpea plants exhibited augmented cadmium content after both short and long term Cd exposures. An increase by 17, 52, 15, 6, 11 and 24-folds in Cd content comparing to control plants was found in root, nodules, stem, leaves, pod covering and seed, respectively, under long term Cd stress while these the short term stress resulted in a lesser accumulation of Cd in different plant parts. Most of Cd was retained in roots and nodules of Cd treated plants. The treatments with NO donor during short and long term Cd stressws promoted improvement and reduced the Cd content in different plant parts.

STOMATAL CONDUCTANCE

Lead stress significantly reduced stomatal conductance by 39% compared to control. Nonetheless, the seeds soaking in SNP raised stomatal conductance under lead stress. 0.5 and 1 mM concentrations of SNP under Pb stress increased stomatal conductance by 64 and 32%, respectively, as compared to Pb treatment alone (Figure 3). Moreover, in this study we also observed that Pb stress elevated ABA content (Figure 1) which caused stomatal closure. In fact,

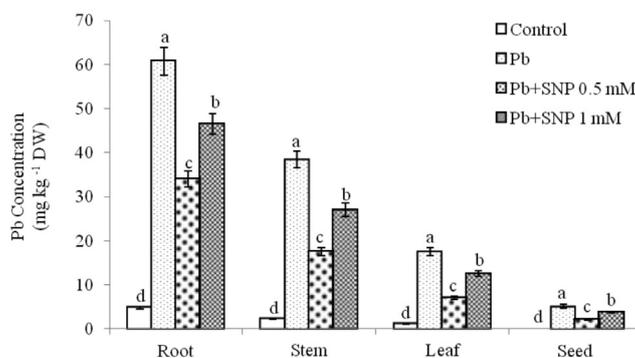


FIGURE 2. Effect of Pb (200 mg kg⁻¹ soil) and SNP (0.5 and 1 mM as NO donor) on Pb concentration of different plant parts of cowpea. Different letters in each plant part indicate significant differences at $p \leq 0.05$ level using Tukey's test. Error bars show SE

there was a negative correlation between ABA content and stomatal conductance. Stomatal movement is controlled by ABA. Lead ions causes a large accumulation of ABA in roots and aerial plant parts (Atici et al. 2005; Cencki et al. 2010) which leading to stomatal closure. Stomatal closure strongly limits gas exchanges and water losses by transpiration. Various mechanisms have been suggested for the Pb-induced decline in transpiration rate and water content. Pb treatment causes growth retardation, which results in a reduced leaf area, the major transpiration organ (Iqbal & Mushtaq 1987). Guard cells are generally smaller in size in plants treated with lead. Pb lowers the level of compounds that are associated with maintaining cell turgor and cell wall plasticity and thus lowers the water potential within the cell. Metal ions including Pb increase the content of ABA and induce stomatal closure (Sharma & Dubey 2005). We found that exogenous NO decreased ABA level and thus partially increased stomatal conductance under Pb stress. Likewise, He et al. (2005) showed that UV-B induced stomatal closure, which was mediated by NO and H₂O₂, and the generation of NO was caused by a NOS-like activity. On the other hand, Garcia-Mata and Lamattina (2001) showed that exogenous NO reduced transpiration and induced stomatal closure in several species. ABA induces the synthesis of NO in guard cells, NO induces stomatal closure and either scavenging of NO or inhibition of NO synthesis reduces ABA-induced stomatal closure. These data indicate that NO synthesis is a

critical component of ABA-induced stomatal closure (Neill et al. 2003). These contrary results for effect of exogenous NO on stomatal movement show that this impact is depending on NO concentration, method of application, plant species, type and severity of stress.

LEAF AREA

The leaf area of cowpea plants markedly was declined by 61% at Pb stress conditions compared to the control. Application of SNP improved leaf area under lead toxicity. 0.5 and 1 mM concentrations of SNP under Pb stress elevated leaf area by 101 and 55%, respectively, as compared to Pb treatment alone (Figure 4). Similar to our results, Elzbieta and Miroslawa (2005) reported that under lead stress, leaf surface area of soybean decreased, led to reduced transpiration. Bhardwaj et al. (2009) also found that leaf area of *Phaseolus vulgaris* showed significant decline with increase in Pb concentrations. Jhanji et al. (2012) revealed that Cd toxicity declined leaf area plant (51%) of *Brassica napus* as compared to the control but exogenous application of NO improved this trait.

SEED YIELD

A noticeable decrease in seed yield was recorded in the Pb treatment resulted in a decline by 56% as compared to control. SNP presoaking increased seed yield under

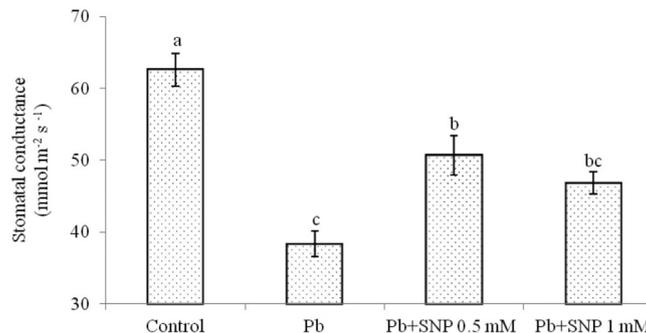


FIGURE 3. Effect of Pb (200 mg kg⁻¹ soil) and SNP (0.5 and 1 mM as NO donor) on stomatal conductance of cowpea. Different letters indicate significant differences at $p \leq 0.05$ level using Tukey's test. Error bars show SE

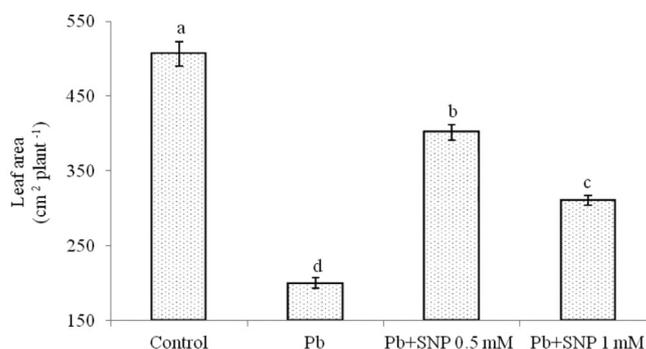


FIGURE 4. Effect of Pb (200 mg kg⁻¹ soil) and SNP (0.5 and 1 mM as NO donor) on leaf area of cowpea. Different letters indicate significant differences at $p \leq 0.05$ level using Tukey's test. Error bars show SE

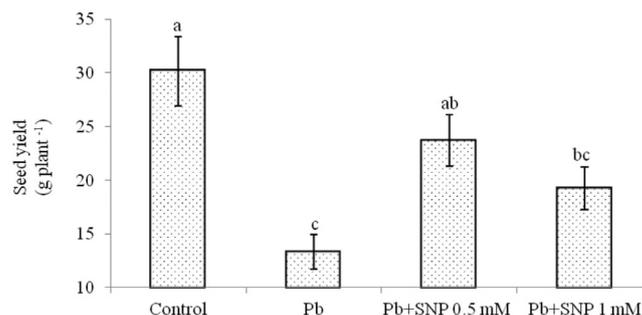


FIGURE 5. Effect of Pb (200 mg kg⁻¹ soil) and SNP (0.5 and 1 mM as NO donor) on seed yield of cowpea. Different letters indicate significant differences at $p \leq 0.05$ level using Tukey's test. Error bars show SE

lead stress. 0.5 and 1 mM concentrations of SNP under Pb stress raised seed yield by 79 and 44%, respectively, compared to the Pb treatment alone (Figure 5). According to our observations in the present study, lead toxicity reduced promoting phytohormone (IAA, cytokinin and GA₃) level, raised ABA content, increased lead uptake and translocation, decreased stomatal conductance and leaf area of cowpea plants resulted in reduced seed yield. Reducing crop yield under Pb stress has been reported by other researchers (Balba et al. 1991; Chatterjee et al. 2004; Hussain et al. 2006). Similarly, Jhanji et al. (2012) and Kumari et al. (2010) found that in chickpea and *Brassica napus* Cd toxicity adversely affected seed yield but exogenous NO improved this trait.

CONCLUSION

The Pb treatment resulted a marked increase in Pb level in all the plant parts. Lead stress decreased promoting phytohormone (IAA, cytokinin and GA₃) content but increased ABA level. Moreover, Pb stress declined stomatal conductance, leaf area and consequently seed yield of cowpea. Exogenous application of NO noticeably diminished the lead toxicity by improving the leaf area, stomatal conductance and seed yield. NO increased Pb tolerance by lowering Pb uptake and translocation, enhancing the promoting phytohormone level and reducing ABA content. 0.5 mM of SNP (NO donor) was more effective in improving all measured traits under lead stress. It can be expected that NO would be a profitable tool in reducing Pb toxicity.

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