Ecotoxicology and Environmental Safety 102 (2014) 25–33

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Hydrogen sulfide alleviates lead-induced photosynthetic and ultrastructural changes in oilseed rape

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ARTICLE INFO

Article history:
Received 2 October 2013
Received in revised form
9 January 2014
Accepted 10 January 2014
Available online 1 February 2014

Keywords:
Plant growth
Root morphology
Lead
Ultrastructural changes
Hydrogen sulfide
Brassica napus L.

ABSTRACT

The role of hydrogen sulfide (H_2S) in alleviating lead (Pb) induced stress in oilseed rape (*Brassica napus* L.) was studied under laboratory conditions. Plants were grown hydroponically in greenhouse conditions under three levels (0, 100, and 400 μM) of Pb and three levels (0, 100 and 200 μM) of H_2S donor, sodium hydrosulfide (NaHS). Application of H_2S significantly improved the plant growth, root morphology, chlorophyll contents and photosynthetic activity in leaves of *B. napus* under Pb stress. Moreover, exogenously applied H_2S significantly lowered the Pb concentration in shoots and roots of plants under Pb stress. The microscopic examination indicated that application of exogenous H_2S enabled a clean mesophyll cell having a well developed chloroplast with thylakoid membranes and starch grains. A number of modifications could be observed in root tip cell i.e. mature mitochondria, long endoplasmic reticulum and golgibodies under combined application of H_2S and Pb. On the basis of these findings, it can be concluded that application of exogenous H_2S has a protective role on plant growth, net photosynthesis rate and ultrastructural changes in *B. napus* plants under high Pb exposures.

1. Introduction

Agricultural soil pollution with toxic metals is a well known global concern since the establishment of industrial revolution. These metals pose a risk to human health and become environmental threats due to their profusion as low solubility, contaminants and the classification as carcinogenic and mutagenic. A wide range of heavy metals has been reported in different biota, one of them is lead (Pb), which originates from various sources including paints, pesticide production, gasoline, Pb smelting and refining etc. Pb is a toxic heavy metal, induces adverse effects in plants on a number of physiological processes such as recession in seed germination, seedling development, root elongation, chlorophyll synthesis, transpiration, lamellar organization in the chloroplast, and cell division (Krzeslowska et al., 2009; Gupta et al., 2009; Maestri et al., 2010). Pb contamination has highly deleterious effects on growth and yield of plants (Gopal and Rizvi, 2008; Islam et al., 2007).

Heavy metals can damage and pose significant changes in the lipid composition of cell membrane (Ali et al., 2013a; Piotrowska et al., 2009). Plant growth retardation under Pb exposure may be due to nutrient imbalance, disturbed photosynthesis, obstruction of the electron transport system and inadequate concentration of carbon dioxide because of stomatal closure (Gopal and Rizvi, 2008; Islam et al., 2008; Romanowska et al., 2006; Qufei and Fashui, 2009). It is well known that Pb inhibits the nutrient uptake, net photosynthetic rate, respiration and causes damage to cell membrane (Sharma and Dubey, 2005). Reduction in net photosynthesis rate is a well-known indicator of Pb-toxicity (Xiong et al., 2006). Photosynthetic activity and photosynthetic pigments are directly affected by metal ions, resulting in the reduction of carbon utilization and respiration process (Sanita di Toppi and Gabbrielli, 2002). Heavy metal toxicity retards the photosynthesis rate by disturbing plant water balance, stomatal conductance, and CO_2 availability or chloroplast organization (Vrettors et al., 2001; Ali et al., 2013a).

Pb accumulation in the soils affects plants primarily through their root systems. It has been reported that roots can take up 3–50 times more Pb than leaves (Wozny et al., 1995). Previously, it is also stated that Pb considerably inhibited the root elongation in *Mesquite* (Arias et al., 2010). Although several studies were focused on the root elongation and biomass; however, a little information is available concerning Pb-toxicity and root morphology (Islam et al., 2007). Recently, Jiang and Liu (2010) reported that exposure of Pb for 72 h to *Allium sativum* roots prompted ultrastructural modifications i.e., mitochondrial swelling, loss of cristae, vacuolization of endoplasmic reticulum, dictyosomes and impaired the lamellar organization in the

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0147-6513/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2014.01.013

chloroplast, and inhibition of cytokinesis leading to binucleate cells (Krzeslowska et al., 2009; Wierzbicka 1989). Among their cytotoxic activities, heavy metals cause oxidative stress (Dixit et al., 2001), which are responsible for the structural and ultrastructural damage to mitochondria, chloroplast membranes (Mittler, 2002), and photosynthetic apparatus through cellular accumulation of reactive oxygen species.

Hydrogen sulfide (H_2S) is a colorless gas with foul odor of rotten eggs and classified as the third gaseous transmitter after nitric oxide (NO) and carbon monoxide (CO) (Wang, 2002). More recent evidences have been proposed that CO is also involved in different biological processes in plants such as root formation (Cao et al., 2007) and protection against oxidative damage induced by salinity (Huang et al., 2006; Xu et al., 2006) and mercury (Han et al., 2007). In plants, H_2S has been considered as an emerging signaling molecule, which regulates different physiological processes in plants (Zhang et al., 2008). It has been reported that maximum production of H_2S resulted from NaHS as compared to other sulfur-containing chemicals (Chen et al., 2011). Lately, it was perceived that H_2S plays a critical role in alleviating the negative effect of aluminum stress in wheat and barley (Zhang et al., 2008; Dawood et al., 2012). H_2S possesses the ability to induce adventitious rooting in different plants (Zhang et al., 2009). It could be applied for the tolerance of plants to abiotic stresses, such as boron (Wang et al., 2010) and aluminum (Zhang et al., 2010). Moreover, Alvarez et al. (2007) interestingly found that biogenic H_2S produced the sulfides which reacted with metal ions resulted into metal precipitation which ultimately can increase the unavailability of metal ions to plants.

Oilseed rape (*Brassica napus* L.) is grown throughout the world for edible oil production. *Brassica* species are generally considered tolerant to heavy metals due to their fast growth, higher biomass and ability of heavy metal absorption (Momoh and Zhou, 2001; Meng et al., 2009). Under heavy metals stress environment, plants employ different strategies against the metal toxicity through specific physiological mechanisms (Papazoglou et al., 2005). Considering the importance of *B. napus*, the present study was carried out to test the hypothesis that H_2S has the ameliorating role on plant growth, photosynthesis, Pb uptake, and ultrastructural damages under Pb stress in oilseed rape.

2. Materials and methods

2.1. Plant growth conditions

Seeds of oilseed rape (*Brassica napus* L. cv. ZS 758) were obtained from the College of Agriculture and Biotechnology, Zhejiang University. The seeds were grown in plastic pots ($170 \times 220\ mm^2$) filled with peat moss. At five leaf stage, seedlings with uniform size were selected and transferred into plate holes on plastic pots having plates with hole (six plants per pot) containing a half strength Hoagland nutrient solution (Arnon and Hoagland, 1940), in which KH_2PO_4 concentration was adjusted to 10 μmol/L in order to prevent precipitation of lead (Pb). The composition of Hoagland nutrient solution was as follows (in μmol/L): 3000 KNO_3, 2000 $Ca(NO_3)_2 \cdot 4H_2O$, 1000 $MgSO_4 \cdot 7H_2O$, 10 KH_2PO_4, 12 $FeC_6H_6O_7$, 500 H_3BO_3, 800 $ZnSO_4 \cdot 7H_2O$, 50 $Mncl_2$, 300 $CuSO_4 \cdot 5H_2O$, 100 Na_2MoO_4. The pH of solution was maintained at 5.5 with 1 M solution of NaOH or HCl. Aeration was given continuously through air pump in the nutrient medium. Nutrient solution was changed after every four days. The light intensity was in the range of 250–350 $\mu mol\ m^{-2}\ s^{-1}$, temperature was recorded 16–20 °C and the relative humidity was approximately 55–60%.

After acclimatization period of 7 days, Pb as $Pb(NO_3)_2$ and H_2S as NaHS were added into full strength Hoagland solution making 9 treatments: (1) control (Ck), (2) 100 μM $Pb(NO_3)_2$ alone, (3) 400 μM $Pb(NO_3)_2$ alone, (4) 100 μM NaHS alone, (5) 200 μM NaHS alone, (6) 100 μM $Pb(NO_3)_2$+100 μM NaHS, (7) 400 μM Pb $(NO_3)_2$+100 μM NaHS, (8) 100 μM $Pb(NO_3)_2$+200 μM NaHS, and (9) 400 μM Pb $(NO_3)_2$+200 μM NaHS. The treatment concentrations were based on pre-experimental studies, in which several lower and higher levels of metal used, i.e., 100, 200, 300, 400, 600 and 1000 μM of Pb. The NaHS was purchased from Sigma (St. Louis, MO, USA) and used as the exogenous H_2S donor as described previously by Chen et al. (2011). They applied a series of sulfur- and sodium-containing chemicals including NaHS, Na_2S, Na_2SO_4, Na_2SO_3, $NaHSO_4$, $NaHSO_3$, and NaAC on *Spinacia oleracea* seedlings and concluded that H_2S rather than other sulfur-containing compounds or sodium was responsible for the increase in plant growth in *S. oleracea* after NaHS treatment. Moreover, it was also found that H_2S contents generated from NaHS were the highest among other chemicals and previously, NaHS was also used as H_2S donor under cadmium and boron stress respectively (Chen et al., 2011; Zhang et al., 2011; Li et al., 2012; Wang et al., 2010). Fifteen days after treatment, all morphological data and photosynthetic parameters were measured. Samples for microscopic studies of leaf mesophyll and root tip were collected as described below.

2.2. Morphological parameters

After 15 days of treatment, plants were harvested and separated into leaf, stem and root. The plant height, stem and root length as well as leaf area of randomly selected six plants per treatment were measured. The root surface area, volume, diameter and number of root tips of randomly selected six plants per treatment were determined using root automatism scan apparatus (MIN MAC, STD1600$^+$), equipped with WinRHIZO software offered by Regent Instruments Co.

2.3. Photosynthetic parameters

Photosynthetic gas exchange parameters were analyzed 15 days after treatment by LiCor-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Topmost fully expanded leaf after 2 h of acclimatization in a growth cabinet, at a temperature of 18 °C under a light intensity of 1000 $\mu mol\ m^{-2}\ s^{-1}$ and relative humidity of 60%, was sampled, and photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate were measured. The total eight readings per treatment were taken from randomly selected plants (Zhou and Leul, 1998). A chlorophyll meter (Minolta Co. Ltd., Japan) was used to take SPAD values of the fully expanded functional leaves (the 4th from the apex), which provided a rapid, accurate, and non-destructive estimate of leaf chlorophyll content. For this purpose, a total of 20 readings per treatment were taken from randomly selected plants to determine chlorophyll content according to Wu et al. (1998). The maximal photochemical efficiency of PSII (Fv/Fm, the ratio of variable fluorescence to maximal fluorescence) was synchronously measured using a portable pulse-modulated fluorometer (FMS-2 Hansatech Instruments Ltd., England).

2.4. Estimation of Pb concentration

For determination of Pb concentration in shoots and roots, samples were dried at 65 °C for 24 h, and then ashed in muffle furnace at 550 °C for 20 h. After that ash was incubated with 31% HNO_3 and 17.5% H_2O_2 at 70 °C for about 2 h, and dissolved in distilled water. Pb concentration in the digest was determined using an atomic absorption spectrophotometer (AA-6800, Shimadzu Co. Ltd., Japan).

2.5. Determination of relative electrolyte leakage (REL)

Plasma membrane integrity in roots was assessed in terms of relative electrolyte leakage (REL). Root tissues (100 mg) were cut into small pieces and vibrated for 30 min in deionized water followed by measurement of conductivity of bathing medium (EC_1). The samples were again boiled for 15 min and second conductivity was measured (EC_2) (Wang and Yang, 2005). Total electrical conductivity was calculated by using the following formula.

$$REL\ (\%) = (EC_1/EC_2) \times 100.$$

2.6. Transmission electron microscopy

After 15 days of treatment, topmost leaf fragments without veins and root tips (8–10 each per treatment) were collected from randomly selected plants and then fixed overnight in 4% glutaraldehyde (v/v) in 0.1 M PBS (Sodium Phosphate Buffer, pH 7.4) and cleansed three times with the same PBS. Samples were post-fixed in 1% OsO_4 (osmium (VIII) oxide) for 1 h, washed three times in 0.1 M PBS (pH 7.4) with 10 min intermission between each washing. After interval for 15–20 min, the samples were dried in a graded series of ethanol (50%, 60%, 70%, 80%, 90%, 95%, and 100%) and at the end washed by absolute acetone for 20 min. The samples were then infiltrated and embedded in Spurr's resin overnight. After heating at 70 °C for 9 h, ultra-thin sections (80 nm) of specimens were prepared and mounted on copper grids for viewing by a transmission electron microscope (JEOL TEM-1230EX, Japan) at an accelerating voltage of 60.0 kV.