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PLANT GROWTH, DEVELOPMENT AND CHANGE IN GSH LEVEL IN SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) EXPOSED TO COPPER AND LEAD

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Abstract: The effects of exposure to heavy metals, copper (Cu) and lead (Pb) in the soil, separately and in combination, were examined in Safflower (*Carthamus tinctorius* L.). Plant growth and development, GSH level and *GSH2* expression at seedling, branching, and flowering stages were studied. Cu at lower concentrations had a stimulating effect on seedling height and root length. A significant positive correlation was observed between heavy metal concentrations and inhibition of plant growth. Plant height, root length and lateral root numbers decreased progressively with increasing concentrations of Cu and Pb. Except at the seedling stage, the metal mixture elicited a synergistic effect on safflower growth and development. The GSH content was significantly reduced in both safflower roots and leaves at increased concentrations of heavy metals, with the exception of the treatment with a low concentration of Cu that resulted in a slightl increase in GSH content at the seedling and branching stages. RT-PCR analysis revealed a negative correlation between *GSH2* expression levels and metal concentration. Short exposure to low concentrations of Cu induce an increase in GSH synthesis to preserve normal plant growth, whereas prolonged exposure and large Cu and Pb concentrations affect the GSH metabolic chain, and are severely toxicity. The findings obtained in this study enhance our understanding of the role of the GSH pool in the response of plants to heavy metal-induced stress, and serve as a basis for improved cultivation of safflower.

Key words: Safflower; copper; lead; GSH; plant growth and development

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INTRODUCTION

Agricultural soils in many parts of the world are contaminated by heavy metals in varying degrees, and heavy metal pollution of agricultural soils is one of the most serious ecological problems worldwide (Yadav 2010; Singh et al., 2007). Studying the effects and mechanisms of heavy metal stress is beneficial for agricultural production. Copper (Cu) is a heavy metal and an essential micronutrient that is critical in maintaining normal metabolism in higher plants. Although it is involved in a wide range of biochemical and physiological processes, an accumulation of excess Cu causes injury on the cellular level by forming reactive oxygen species (ROS) in plant cells (Upadhyay and Panda, 2009, Nzengue et al., 2011). Lead (Pb) is one of the most toxic heavy metals and is a human carcinogen. It has adverse effects on plant morphology, growth and photosynthetic processes. High levels of Pb can also inhibit enzymatic activities, alter membrane permeability, disrupt mineral nutrition (Sharma and Dubey 2005) and induce oxidative stress by increasing ROS production in plant cells (Reddy et al., 2005). To combat the oxidative damage induced by heavy metal stress, plants have an antioxidant defense system that may involve GSH (glutathione) (Speisky et al., 2009; Estrella-Gómez et al., 2012).

GSH is a tripeptide formed by a peptide bond condensation of L-glutamic acid, L-cysteine and glycine (Wachter et al., 2005). It is synthesized by two sequential reactions catalyzed by γ-glutamylcysteine synthetase (GSH1) and GSH synthetase (GSH2) (Okuma et al., 2011; Estrella-Gómez et al., 2012). GSH is a major intracellular thiol compound (Iwasaki et al., 2009) that plays important roles in many biological processes, such as intracellular reduction-oxidation metabolic cycles, transport, protein synthesis (Ensafi et al., 2013) and elimination of ROS (Foyer and Noctor 2005; Mullineaux and Rausch 2005; Morris et al., 2013) in plants. The glutathione molecule contributes to the regulation of the intracellular redox environment and the detoxification of xenobiotics caused by an excess of metals, including Cu and Pb, and it also functions as a crucial intracellular antioxidant (Roušar et al., 2012; Menon and Board, 2013). GSH1 and GSH2 are important enzymes for GSH synthesis and accumulation. *GSH1* and *GSH2* mRNAs are increased by conditions that require enhanced GSH abundance for metabolic functions, such as heavy metal sequestration (Maughan and Foyer, 2006; Xiang and Oliver, 1998). Recently, Blazhenko et al. (2014) reported that *GSH2* transcription increased in the yeast *Hansenula polymorpha* under cadmium (Cd) treatment. However, unlike the extensive study of *GSH1* (Rausch et al., 2007), *GSH2* was less studied in plants, especially in safflower.

Safflower (*Carthamus tinctorius* L.) is a traditional medicinal plant and represents a new type of oilseed crop and industrial resource. To date, most studies have focused on the genetic resource (Patel et al., 1989; Yang et al., 2007), cultivation techniques (Kizel, 2002), chemical constituents (Takahashi et al., 1984; Zhang et al., 1997) and pharmacology (Chu et al., 2006; Jun et al., 2011) of safflower. Studies on heavy metal toxicity in safflower are limited (Nosrati et al., 2013). The levels of Cu and Pb that induce toxicity or growth alterations are not known. Moreover, antioxidants, such as GSH, and related gene expression responses to heavy metal stress are also not known. In this study, a greenhouse pot experiment was conducted to evaluate the effect of Cu and Pb on safflower development, GSH level and *GSH2* gene expression. Safflowers were grown in soil with Cu, Pb, or both. We focused on the morphological, biochemical and molecular

properties, such as plant height, root length, lateral root number, GSH content and *GSH2* expression, to evaluate the effects of Cu and Pb on plant growth and development of safflower.

MATERIALS AND METHODS

Plant materials and cultivation methods

Cu was added to soil (Xinxiang Forint Health Food Co., Ltd., Xinxiang, China) as an aqueous mixture of the $CuCl_2$ in the following concentrations: 50 μg/g (Cu50; 50 μg Cu/g of soil), 100 μg/g (Cu100), 400 μg/g (Cu400) and 800 μg/g (Cu800). Pb was added to the soil in a similar manner in the following concentrations of $Pb(NO_3)_2$: 300 μg/g (Pb300), 500 μg/g (Pb500), 800 μg/g (Pb800) and 1000 μg/g(Pb1000). The following concentrations were used for the Cu-Pb combinations: Cu 50 μg/g and Pb 300 μg/g (Cu50 + Pb300), Cu 100 μg/g and Pb 500 μg/g (Cu100 + Pb500), Cu 400 μg/g and Pb 800 μg/g (Cu400 + Pb800), and Cu 800 μg/g and Pb 1000 μg/g (Cu800 + Pb1000). Plastic pots (80×24×17 cm^3) were filled with 7.5 kg of the treated soil samples. Untreated soil was used as control. All treatments were replicated three times.

Safflower seeds (Variety 'Wei honghua', Xinxiang Forint Health Food Co., Ltd.) were surface-sterilized with 0.1% (w/v) $HgCl_2$ for 5 min and then washed thoroughly with sterile water. Sterilized seeds were germinated in Petri plates on sterile wet paper towels at 25°C in the dark for 3 days. Twenty-three-day-old germinated seeds were transferred to each of the pots containing soil treated with Cu, Pb or Cu-Pb. The experiment was conducted at a standard long day light (16 h) at 25°C-35°C during the day, and at 10°C-18°C during the night (8 h), at 65±5% relative humidity. The plants were cultivated for 240 days from 15 October 2011 to 15 June 2012. During cultivation, controlled irrigation using water was scheduled according to tensiometer

Table 1. Primers used in this study

PCR	Primer name	Sequence (5′-3′)
conserved regions	*GSH2*-F	GATCCGTGTTAGCTTGGATGG
	GSH2-R	ATGTTGTTWCCTCCGCCTTCTC
3′-RACE	3′-GSP1	CTGGCTGAAATTGATGCAC
	3′-Outer primer	TACCGTCGTTCCACTAGTGATTT
	3′-GSP2	TTCAGCAGGAGCTTGCAAAACCG
	3′-Inner primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG
5′-RACE	5′-Outer primer	CATGGCTACATGCTGACAGCCTA
	5′-GSP1	GCTCCTATGAAGCTCGCTGAC
	5′-Inner primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
	5′-GSP2	TCCTGTAGAAACTTGCCATCCAAGCTAACACGG
Semi-quantitative RT-PCR	*actin*01	TTACCGTAAAGGTCCTTCCTGAT
	*actin*02	AGCTTCGTGTTGCTCCTGAAGA
	LEFT	ATCCGTGTTAGCTTGGATGGC
	RIGHT	GCCAATGTTGGTCATACATG

measurements to maintain soil moisture content within field capacity. In the potted processing experiments, the growth of safflower was divided into seedling (approximately 60 days after transplantation), rosette (approximately 100 days after transplantation), elongation (approximately 140 day after transplantation), branching (approximately 170 days after transplantation), flowering (approximately 210 days after transplantation) and mature (about 240 days after transplantation) stages. We selected three stages, namely, the seedling, branching and flowering stages, for the measurement of the parameters of plant growth and GSH content.

Determination of plant height, root length and lateral root number

Plants were harvested at the end of each selected stage, and the height, root length and lateral root number were determined. Fifteen plants were evaluated for each treatment.

Assays of glutathione content

The GSH content was determined using the method of Ellman (1959) with modifications. In brief, 0.2 g of safflower samples (roots or leaves) were rinsed in cold water (0°C) and the excess water was removed using a filter paper. Each sample was grounded in 3 mL of 5% trichloroacetic acid solution by centrifugation for 20 min at 4°C; 0.1 mL of the resulting supernatant was mixed with 4.4 mL of PBS (0.1 mol/L) and 0.5 mL of 5.5-dithiobis (2-nitro-benzoic acid; 0.04% w/v). The samples were incubated at 30°C for 5 min in the dark. The absorbance was read at 412 nm using a Unico spectrophotometer (UV-4802). The concentration of GSH was from the calibration curve obtained with commercial standards (Sigma, Taufkirchen, Germany).

Statistical analysis

Data were subjected to one-way ANOVA using SPSS version 18.0 (PASW Statistics for Windows, 2009, Chicago: SPSS Inc.). Separate analyses were performed for each sampling, and significant differences (p <0.05 or p <0.01) were determined between the metal treatments and the control.

Safflower total RNA isolation and full-length GSH2 gene cloning

Total RNA was isolated from the control and heavy-metal-treated safflower seedlings using the Super Purified Total RNA Rapid Extraction Kit (Aidlab Biotech, Beijing, China). RNA samples were treated with RNase-free DNase I (Takara, Dalian, China) at 37°C for 30 min. The first-strand cDNA was synthesized from 2 μg of RNA template with TUREscript 1st Strand cDNA Synthesis Kit (Aidlab) according to the manufacturer's protocols with oligo $(dT)_{18}$ as a primer. The cDNA samples were used as templates in the subsequent degenerate PCR and semi-quantitative RT-PCR.

Degenerate primers were designed from the conserved amino acid regions of the GSH2 protein (Table 1). PCR was performed by denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 1 min with additional elongation at 72°C for 10 min after the last cycle. Amplified products were cloned into the vector pMD18-T (TaKaRa) and transformed into competent *Escherichia coli* DH5α. Positive clones were screened from Amp^+/LB plates containing IPTG/X-Gal and then sent to SinoGenoMax (Beijing, China) for DNA sequencing.

The full-length *GSH2* cDNA was obtained by rapid amplification of cDNA ends (RACE) tech-

nology. Based on the conserved regions of the cloned *GSH2* gene, gene-specific primers (Table 1) were designed for 3′-RACE and 5′-RACE. Reverse transcription reactions were performed with 3 μg-5 μg of total RNA to produce 3′- and 5′-cDNA using the RACE Core Set (TakaRa) according to the manufacturer's instructions. The RACE products were cloned into the pMD18-T vector and sequenced (SinoGenoMax).

Semi-quantitative PCR analysis of GSH2 expression

Total RNA was extracted from the control and different concentrations of metal-treated safflower leaves in the seedling, branching and flowering stages. RNA samples were treated with RNase-free DNase I, and the first-strand cDNA was synthesized from 2 μg of RNA. Table 1 lists the PCR

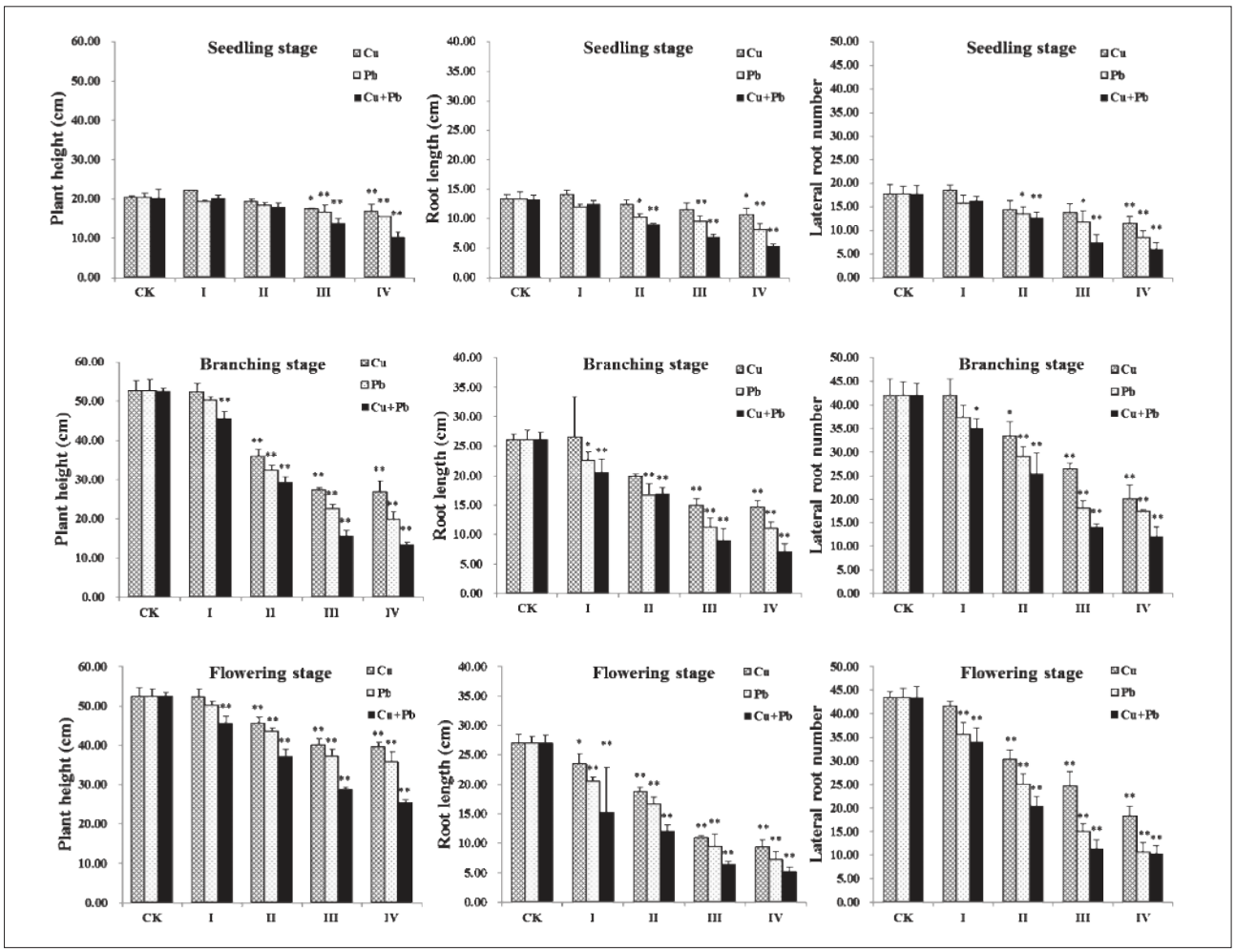

Fig. 1. Effects of Cu, Pb and their combination on plant height, root length, and lateral root number in safflower. Plant height, root length, and lateral root number were analyzed from control and heavy metal-treated safflowers at seedling, branching and flowering stages. The X axis represents the concentrations of Cu and Pb (μg/g) as follows: Cu (CK: Cu0; I: Cu50; II: Cu100; III: Cu400; IV: Cu800), Pb (CK: Pb0; I: Pb300; II: Pb500; III: Pb800; IV: Pb1000), and Cu + Pb (CK: Cu0 + Pb0; I: Cu50 + Pb 300; II: Cu100 + Pb500; III: Cu400 + Pb800; IV: Cu800 + Pb1000). One-way ANOVA was used to analyze the significant difference, and asterisk represents the significant difference between metal-treated samples and the control (*P<0.05; **P<0.01). Values are expressed as mean ± SD based on three independent experiments and bars indicate SD.

primers used for detecting the safflower *GSH2* gene. The housekeeping *actin* gene was used as the control in the RT-PCR reactions, and the primers are listed in Table 1 (Sugiyama et al., 2003). The reaction included denaturation at 95°C for 5 min, followed by 35 cycles (25 cycles for *actin*) of 30 s at 95°C, 30 s at 50°C, 1 min at 72°C and then 10 min at 72°C. The amplification products were separated by electrophoresis on 1.5% (w/v) agarose gel and visualized by a UV imaging system.

RESULTS

Effects of different metal treatments on safflower growth and development

To examine safflower growth and development response to Cu and Pb, we determined the plant height, root length and lateral root number. Comparisons between metal-treated and untreated plants at seedling, branching and flowering stages were carried out (Fig. 1). Results showed that plant height and root length increased after exposure to a low concentration of Cu (50 μg/g) at the seedling stage; however, the lateral root number of safflower plants showed no significant change compared with the control at the same stage. Safflower seedlings exposed to high Cu concentrations (≥400 μg/g) showed evident signs of growth inhibition. Plant height and lateral root number remarkably decreased when the concentration of Cu reached 100 μg/g, and root length significantly decreased when the concentration of Cu was more than 400 μg/g at the branching stage. Root length and lateral root number decreased after exposure to all of the examined concentrations of Cu (increasing "levels" of Cu stress) at the flowering stage.

Regarding the treatment with Pb, inhibition in plant growth was directly proportional to the Pb concentration in the soil. Root length and lateral root number significantly decreased at the seedling stage when the concentration was greater than 500 μg/g, and plant height significantly decreased when the concentration was 800 μg/g. Root length significantly decreased at the branching stage, even with the lowest Pb concentration (300 μg/g), and plant height and lateral root numbers decreased when the concentration of Pb ≥ 500 μg/g. Root length and lateral root number in the flowering stage were significantly inhibited, even with the lowest concentration of Pb.

As for the stress incurred by the combination of Cu and Pb, plant height, root length and lateral root number were all significantly inhibited in the branching and flowering stages. In addition, the degree of inhibition observed in the treatment with the Cu and Pb combination was larger than in the treatments with Cu or Pb alone. For example, plant height, root length and number of lateral roots decreased by 51.5%, 80.7% and 76.2%, respectively, under combined treatment of Cu (800 μg/g) and Pb (1000 μg/g), and 24.5%, 65.2% and 57.7% under the treatment with 800 μg/g Cu, and 31.8%, 73.3% and 75.4% under the treatment with 1000 μg/g Pb.

Results also showed that the degree of inhibition of safflower growth was directly proportional to the length of exposure to Cu and Pb. For example, root length was reduced by 19.9%, 44.1% and 65.2% in the seedling, branching, and flowering stages, respectively.

Effect of different metal treatments on GSH content in safflower roots and leaves

The GSH contents in the roots and leaves were determined to evaluate the biochemical response of safflower to Cu and Pb. As shown in Fig. 2A,

when the plants were exposed to 100 μg Cu/g and 300 μg Pb/g, the GSH content displayed a significantly (P <0.01) decreasing trend with the increase in metal concentration at all growth stages. Notably, the GSH content increased slightly under the lowest concentration of Cu (50 μg/g) at the seedling and branching stages (P <0.05), suggesting that short exposure to low concentrations of Cu could induce an increase in the GSH content in safflower roots. Furthermore, the effect of Cu-Pb stress on the GSH content of safflower roots was considerably greater than that of Cu or Pb alone. Under the treatment with the highest metal concentrations, at the flowering stage the GSH content of roots was significantly (P <0.01) reduced, by 55.27% (Cu 800 μg/g), 63.91% (Pb 1000 μg/g) and 72.62% (Cu 800 μg/g + Pb 1000 μg/g) as compared to the control.

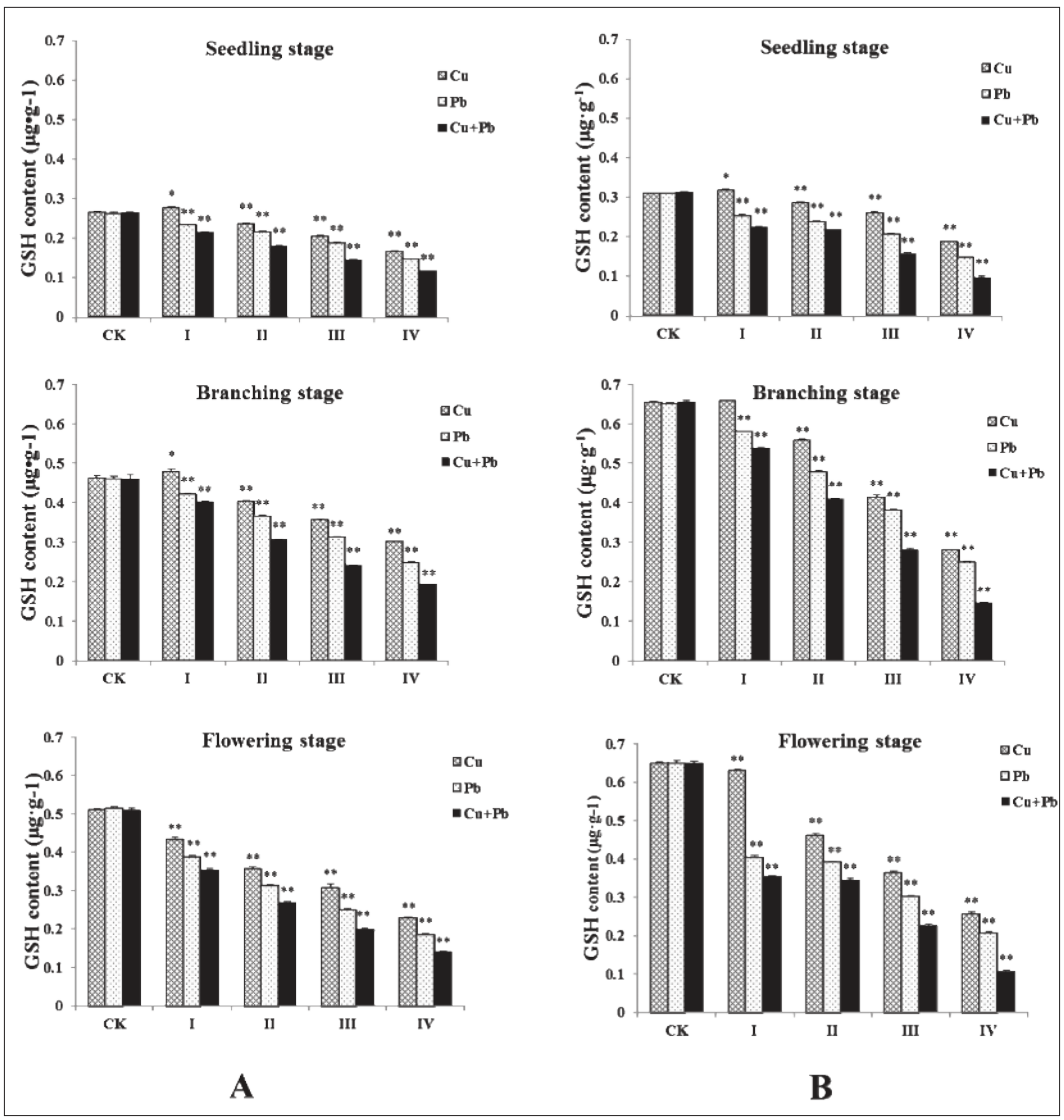

Fig. 2. Effects of Cu, Pb and their combination on the GSH content in (A) roots and (B) leaves of safflower. The GSH content of roots and leaves was analyzed in control and heavy metal-treated safflower at the seedling, branching and flowering stages. The X axis and asterisk are as explained in Fig. 1. Values are expressed as mean ± SD based on three independent experiments and bars indicate SD.

In general, the pattern of changes in the GSH content in safflower leaves at different concentrations of heavy metal was similar to that in safflower roots (Fig. 2B). The GSH content significantly (P <0.01) decreased in the leaves of safflower with increasing heavy metal concentration, except in the treatment with a low concentration of Cu (50 μg/g) at the seedling and branching stages. Interestingly, an increase in GSH content was also observed in safflower leaves under short exposure to low concentrations of Cu. The toxic effect of Cu-Pb on GSH content of safflower leaves was higher than that of Cu or Pb alone, suggesting a collaborative effect of Cu and Pb.

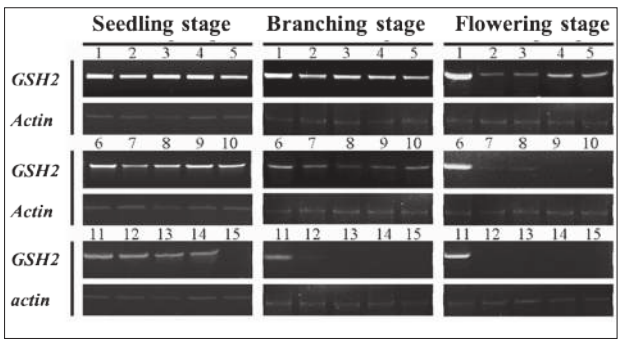

Fig. 3. Expression of the *GSH2* gene in the leaves of safflower exposed to Cu-, Pb- and Cu+Pb-induced stress. The actin gene was amplified by RT–PCR and served as an internal reference. The numbers represent the different concentrations of Cu, Pb, and Cu+Pb (μg/g). 1: CK; 2: Cu50; 3: Cu100; 4: Cu400; 5: Cu800; 6: CK; 7: Pb300; 8: Pb500; 9: Pb800; 10: Pb1000; 11: CK; 12: Cu50 + Pb300; 13: Cu100 + Pb500; 14: Cu400 + Pb800; 15: Cu800 + Pb1000.

Gene expression analysis of safflower GSH2 in response to different heavy metal treatments

The expression of *GSH2* was analyzed to evaluate further the relationship between changes in GSH levels and gene expression. The 1263 bp transcript sequence of *GSH2* was obtained by RACE-PCR. RT-PCR analysis showed a negative relationship between *GSH2* expression levels and metal concentration. In the control and Cu-stressed leaves of safflower seedlings, *GSH2* expression levels did not change, with the exception of leaves from plants exposed to 800 μg/g Cu. However, the expression levels of *GSH2* in leaves decreased considerably at the branching and flowering stages (Fig. 3). Furthermore, the *GSH2* gene of safflower leaves at the branching and flowering stages was barely expressed in plants that were exposed to the Cu-Pb combination.

DISCUSSION

Heavy metal contamination has adverse effects on plant growth and development, such as reduced root length, leaf area, plant height, dry weight, and a decline in photosynthesis (Vinit-Dunand et al., 2002; Oncel et al., 2000; Bradshaw and Chadwick, 1980). The present study revealed that short exposure to low concentrations of Cu and Pb had a minimal influence on safflower growth and development, whereas prlonged exposure and high concentrations exerted adverse effects. In the present study, both Cu and Pb were identified as effective elements for inhibiting safflower growth. A positive correlation was observed between the degree of toxicity and heavy metal concentration. Similar phytotoxic responses to Cu and Pb are observed in many plant species (Munzuroglu and Geckil, 2002; MacFarlane and Burchett, 2002; An et al., 2004; Shu et al., 2014). For example, Munzuroglu and Geckil (2002) studied the inhibitory effects of six metals, including Cu and Pb, on wheat and cucumber, and the results showed that both plants exhibit reduced seed germination rates, root, and hypocotyl or coleoptile length with increasing concentrations of heavy metals. The toxic symptomatology observed is speculated

to be a consequence of tissue damage, alteration of membrane permeability, peroxidation of chloroplast membrane lipids, inhibition of photosynthetic electron transport and disruptions to carbohydrate metabolism and protein synthesis (Droppa and Horvath, 1990; Wozny and Krzeslowska, 1993; MacFarlane and Burchett, 2002). Notably, lower Cu concentrations stimulated plant height and root length in the seedling stage in this study. Shu et al. (2014) also reported that Pb has a stimulating effect on the seedling height and leaf area of *Jatropha curcas* L. at low concentrations. These findings suggest a positive effect of low concentrations of some heavy metals on plant growth.

Different compounds are often found as mixtures in the environment. Soil pollution by combinations of heavy metals has received considerable attention. The exposure of cells to a combination of metals may result in increased permeability of the plasma membrane accompanied by increased cellular uptake and toxicity (MacFarlane and Burchett, 2002). Several studies have been conducted to investigate the combined effects of heavy metals on some plant species. The combination of Pb and Zn was reported to increase toxicity to *Avicennia marina* (grey mangrove) (MacFarlane and Burchett, 2002). Another report showed that Cu, Cd and Pb have a combined effect on *Cucumis sativus* growth and inhibit or enhance the bioaccumulation of individual metals depending on metal combination (An et al., 2004). Interactions between Cu and Pb have been rarely reported. In this study, the combination of Cu and Pb exhibited higher toxicity to safflower growth and development compared to Cu or Pb alone, indicating an interaction between these two metals in transport or a reduction in the efficacy of exclusion from plantGSH is a prominent cellular antioxidant that plays a critical role in membrane protection against free radical damage in most eukaryotic organisms (Rennenberg and Brunold, 1994; Moran et al., 2000). Our results showed that short exposure and low concentrations of Cu could induce an increase in GSH content in safflower roots and leaves. However, when plants were exposed to 100 μg/g Cu and 300 μg/g Pb, the GSH content progressively decreased with increasing heavy metal concentrations. The results were apparently inconsistent with the majority of studies. Increased levels of GSH were observed with increasing Cu or Pb concentrations in plant species, such as *Arabidopsis thaliana* (*Drążkiewicz* et al., 2003), *Sedum alfredii* (Gupta et al., 2010) and *Phanerochaete chrysosporium* (Xu et al., 2014). However, a decline in GSH content in *Brassica napus* roots (Russo et al., 2008) and *Scenedesmus bijugatus* cells (Nagalakshmi and Prasad, 2001) has been reported under Cu stress. A Pb-induced decline in GSH was reported in *Vicia faba*, *Phaseolus vulgaris* (Piechalak et al., 2002) and *Pisum sativum* (Malecka et al., 2009). Furthermore, an unchanged GSH content was observed in the nodules of *Glycine max* under Cd stress (Balestrasse et al., 2001). These findings reveal that GSH levels in plants are metal-dependent (Israr et al., 2011), dose (Kalinowska and Pawlik-Skowronska, 2010), plant cultivars (Cruz de Carvalho et al., 2010) and even tissue type (Russo et al., 2008). García-Giménez et al. (2012) recently reported that GSH might be involved in epigenetic phenomena and the control of nuclear protein degradation by nuclear proteasome. These reports imply that a complex mechanism underlies the GSH response to heavy metal stress. Based on the consistency between *GSH2* expression and changes in GSH content, our results indicated that GSH protects the normal growth of plant under short exposures to low concentrations

of Cu, whereas an excess of Cu, Pb or Cu and Pb may alter the equilibrium between the synthesis and utilization of GSH, probably via the regulation of *GSH2* gene expression. As previously discussed, the relationship between GSH and the stress response is complex, and further studies are necessary.

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Authors' contributions: Shufen Li, Wujun Gao and Longdou Lu conceived and designed the experiments; Shufen Li and Xingpeng Zhao performed the experiments; Chuanliang Deng analyzed the data and Shufen Li and Wujun Gao wrote the paper.

REFERENCES

An, Y. J., Kim, Y. M., Kwon, T. I. and *S. W Jeong* (2004). Combined effect of copper, cadmium, and lead upon *Cucumis sativus* growth and bioaccumulation. *Sci. Total Environ*. **326**, 85-93.

Balestrasse, K. B., Gardey, L., Gallego, S. M. and *M. L. Tomaro* (2001). Response of antioxidant defence system in soybean nodules and roots subjected to cadmium stress. *Aust. J. Plant Physiol*. **28**, 497-504.

Blazhenko, O. V., Kotliarchuk, A. B. and *V. M. Ubyĭvovk* (2014). Transcriptional regulation of the *Hansenula polymorpha GSH2* gene in response to cadmium ion treatment. *Ukr. Biokhim. Zh.* **86**, 75-84.

Bradshaw, A. D. and *J. Chadwick* (1980). The Restoration of Land: The ecology and reclamation of derelict and degraded land Blackwell, Oxford.

Chu, D., Liu, W., Huang, Z., Liu, S., Fu, X. and *K. Liu* (2006). Pharmacokinetics and excretion of hydroxysafflor yellow A, a potent neuroprotective agent from safflower, in rats and dogs. *Planta Med*. **72**, 418-423.

Cruz de Carvalho, M. H., Brunet, J., Bazin, J., Kranner, I., d' Arcy-Lameta, A., Zuily-Fodil, Y. and *D. Contour-Ansel* (2010). Homoglutathione synthetase and glutathione synthetase in drought-stressed cowpea leaves: expression patterns and accumulation of low-molecular-weight thiols. *J. Plant Physiol*. **167**, 480-487.

Drążkiewicz, M., Skórzyńska-Polit, E. and *Z. Krupa* (2003). Response of the ascorbate-glutathione cycle to excess copper in *Arabidopsis thaliana* (L.). *Plant Sci.* **164**, 195-202.

Droppa, M. and *G. Horvath* (1990). The role of Cu in photosynthesis. Crit. Rev. *Plant Sci*. **9**, 111-124.

Ellman, G. L (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophysics* **18**, 70-77.

Ensafi, A. A., Karimi-Maleh, H. and *S. Mallakpour* (2013). A new strategy for the selective determination of glutathione in the presence of nicotinamide adenine dinucleotide (NADH) using a novel modified carbon nanotube paste electrode. *Colloids Surf. B Biointerfaces* **104**, 186-193.

Estrella-Gómez, N. E., Sauri-Duch, E., Zapata-Pérez, O. and *J. M. Santamaría* (2012). Glutathione plays a role in protecting leaves of *Salvinia minima* from Pb^{2+} damage associated with changes in the expression of *SmGS* genes and increased activity of GS. *Environ. Experiment. Bot*. **75**, 188-194.

Foyer, C. H. and *G. Noctor* (2005). Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant Cell Environ*. **28**, 1056-1071.

García-Giménez, J. L., Markovic, J., Dasí, F., Queval, G., Schnaubelt, D., Foyer, C. H. and *F. V. Pallardó* (2013). Nuclear glutathione. *Biochim. Biophys. Acta*. **1830**, 3304-3316.

Gupat, D. K., Huang, H. G., Yang, X. E., Razafindrabe, B. H. N. and M. Inouhe (2010). The detoxification of lead in *Sedum alfredii* H. Is not related to phytochelatins but the glutathione. *J. Hazard. Mater.* **177**, 437-444.

Israr, M., Jewell, A., Kumar, D. and *S. V. Sahi* (2011). Interactive effects of lead, copper, nickel and zinc on growth, metal uptake and antioxidative metabolism of *Sesbania drummondii*. *J. Hazard. Mater*. **186**, 1520-1526.

Iwasaki Y., Saito Y., Nakano Y., Mochizuki K., Sakata O., Ito R., Saito K. and *H. Nakazawa* (2009). Chromatographic and mass spectrometric analysis of glutathione in biological samples. *J. Chromatog. B* **877**, 3309-3317.

Jun, M. S., Ha, Y. M., Kim, H. S., Jang, H. J., Kim, Y. M., Lee, Y. S., Kim H. J., Seo H. G., Lee J. H., Lee S. H. and *K. C. Chang* (2011). Anti-inflammatory action of methanol extract of *Carthamus tinctorius* involves in heme oxygenase-1 induction. *J. Ethnopharmacol*. **133**, 524-530.

Kalinowska, R. and *B. Pawlik-Skowrońska* (2010). Response of two terrestrial green microalgae (*Chlorophyta, Trebouxiophyceae*) isolated from Cu-rich and unpolluted soils to copper stress. *Environ. Pollut*. **158**, 2778-2785.

Kizel, S. (2002). A study on the determination of suitable sowing date of safflower (*Carthamus tinctorius* L.) in Diyarbaki ecological conditions. *Anadolu* **12**, 37-50.

Malecka, A., Piechalak, A. and *B. Tomaszewska* (2009). Reactive oxygen species production and antioxidative defense system in pea root tissues treated with lead ions: the whole roots level. *Acta Physiol. Plant* **31**, 1053-1063.

Maughan, S. and *C. H. Foyer* (2006). Engineering and genetic approaches to modulating the glutathione network in plants. *Physiol. Plantarum*. **126**, 382-397.

MacFarlane, G. R. and *M. D. Burchett* (2002). Toxicity, growth and accumulation relationship of copper, lead and zinc in the grey mangrove *Avicennia marina* (Forsk.) Vierh. *Mar. Environ. Res*. **54**, 65-84.

Menon, D. and *P. G. Board* (2013) A fluorometric method to quantify protein glutathionylation using glutathione derivatization with 2,3-naphthalenedicarboxaldehyde. *Anal. Biochem*. **433**, 132-136.

Moran, J. F., Iturbe-Ormaetxe, I., Matamoros, M. A., Rubio, M. C., Clemente, M. R., Brewin, N. J. and *M. Becana* (2000). Glutathione and homoglutathione synthetases of legume nodules: cloning, expression, and subcellular localization. *Plant Physiol*. **124**, 1381-1392.

Morris, D., Khurasany, M., Nguyen, T., Kim, J., Guilford, F., Mehta, R., Gray D., Saviola B. and *V. Venketaraman* (2013). Glutathione and infection. *Biochim. Biophys. Acta*. **1830**, 3329-3349.

Mullineaux, P. M. and *T. Rausch* (2005). Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. *Photosynthesis Res*. **86**, 459-474.

Munzuroglu, O. and *G. Geckil* (2002). Effects of metals on seed germination, root elongation, and coleoptile and hypocotyl growth in *Triticum aestivum* and *Cucumis sativus*. *Arch. Environ. Contam. Toxicol*. **43**, 203-213.

Nagalakshmi, N. and *M. N. V. Prasad* (2001). Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. *Plant Sci*. **160**, 291-299.

Noctor, G., Gomez, L., Vanacker, H. and *C. H. Foyer* (2002). Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *J. Exp. Bot*. **53**, 1283-304.

Nosrati, S., Asli, E. D. and *A. Pazoki* (2013). Studying the resistance, absorption and accumulation of cadmium in safflower (*Carthamus tinctorius* L.) plant. *Ann. Biol. Res*. **4**, 169-173.

Nzengue, Y., Candéias, S. M., Sauvaigo, S., Douki, T., Favier, A., Rachidi, W. and *P. Guiraud* (2011). The toxicity redox mechanisms of cadmium alone or together with copper and zinc homeostasis alteration: its redox biomarkers. *J. Trace Elem. Med. Biol*. **25**, 171-180.

Okuma, E., Jahan, M. S., Munemasa, S., Hossain, M. A., Muroyama, D., Islam, M. M., Ogawa K., Watanabe-Sugimoto M., Nakamura Y., Shimoishi Y., Mori I. C., and *Y. Murata* (2011). Negative regulation of abscisic acid-induced stomatal closure by glutathione in Arabidopsis. *J. Plant Physiol*. **168**, 2048-2055.

Oncel, I., Keles, Y. and *A. S. Ustun* (2000). Interative effects of temperature and heavy metal stree on the growth and some biochemical compounds in wheat seedlings. *Environ. Pollut*. **107**, 315-320.

Patel, M. Z., Reddi, M. V., Rana, B. S. and *B. J. Reddy* (1989). Genetic divergence in safflower (*Carthamus tinctorius* L.). *Indian J. Genet. Pl. Br*. **49**, 113-118.

Piechalak, A., Tomaszewska, B., Baralkiewicz, D. and *A. Malecka* (2002). Accumulation and detoxification of lead ions in legumes. *Phytochemistry* **60**, 153-162.

Rausch, T., Gromes, R., Liedschulte, V., Müller, I., Bogs, J., Galovic, V. and *A. Wachter* (2007). Novel insight into the regulation of GSH biosynthesis in higher plants. *Plant Biol*. **9**, 565-572.

Reddy, A. M., Kumar, S. G., Jyothsnakumari, G., Thimmanaik, S. and *C. Sudhakar* (2005). Lead induced changes in antioxidant metabolism of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) and bengalgram (*Cicer arietinum* L.). *Chemosphere* **60**, 97-104.

Rennenberg, H. and *C. Brunold* (1994). Significance of glutathione metabolism in plants under stress. *Prog. Bot.* **55**, 142-156.

Roušar, T., Kučera, O., Lotková, H. and *Z. Cervinková* (2012). Assessment of reduced glutathione: comparison of an optimized fluorometric assay with enzymatic recycling method. *Analy. Biochem*. **423**, 236-240

Russo, M., Sgherri, C., Izzo, R. and *F. Navari-Izzo* (2008). *Brassica napus* subjected to copper excess: Phospholipases C and D and glutathione system in signaling. *Environ. Exp. Bot*. **62**, 238-246.

Sharma, P. and *R. S. Dubey* (2005). Lead toxicity in plants. *Braz. J. Plant Physiol*. **17**, 35-52.

Singh, D., Nath, K. and *Y. K. Sharma* (2007). Response of wheat seed germination and seedling growth under copper stress. *J. Environ. Biol*. **28**, 409-414.

Shu, X., Zhang, Q. F. and *W. B. Wang* (2014). Lead induced changes in growth and micronutrient uptake of *Jatropha curcas* L. *Bull. Environ. Contam. Toxicol*. **93**, 611-617.

Speisky, H., Gómez, M., Burgos-Bravo, F., López-Alarcón, C., Jullian, C., Olea-Azar, C. and *M. E. Aliaga* (2009). Generation of superoxide radicals by copper–glutathione complexes: Redox-consequences associated with their interaction with reduced glutathione. *Bioorg. Med. Chem*. **17**, 1803-1810.

Sugiyama, R., Kazama, Y., Miyazawa, Y., Matsunaga, S. and *S. Kawano* (2003). CCLS96.1, a member of a multicopy gene family, may encode a non-coding RNA preferentially transcribed in reproductive organs of *Silene latifolia*. *DNA Res*. **10**, 213*Takahashi, Y., Saito, K., Yanagiya, M., Ikura, M., Hikichi, K., Matsumoto, T.* and *M. Wada* (1984). Chemical constitution of safflor yellow B, a quinochalcone c-glycoside from the flower petals of *Carthamus tinctorius* L. *Tetrahedron Lett*. **25**, 2471-2474.

Upadhyay, R. K. and *S. K. Panda* (2009). Copper-induced growth inhibition, oxidative stress and ultrastructural alterations in freshly grown water lettuce (*Pistia stratiotes* L.). *CR. Biol.* **332**, 623-632.

Vinit-Dunand, F., Epron, D., Alaoui-Sosse, B. and *P. Badot* (2002). Effects of copper on growth and on photosynthesis of mature and expanding leaves in cucumber plants. *Plant Sci*. **163**, 53-58.

Wachter, A., Wolf, S., Steininger, H., Bogs, J. and *T. Rausch* (2005). Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the Brassicaceae. *Plant J*. **41**, 15-30.

Wozny, A. and *M. Krzeslowska* (1993). Plant cell response to Pb. *Acta Soc. Bot. Pol*. **62**, 101-105.

Xiang, C. and *D. J. Oliver* (1998). *Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis. Plant Cell* **10**, 1539-1550.

Xu, P., Liu, L., Zeng, G., Huang, D., Lai, C., Zhao, M., Huang, C., Li, N., Wei, Z., Wu, H., Zhang, C., Lai, M. and *Y. He* (2014). Heavy metal-induced glutathione accumulation and its role in heavy metal detoxification in *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol*. **98**, 6409-6418.

Yadav, S. K. (2010). Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South African J. Bot*. **76**, 167-179.

Yang, Y. X., Wu, W., Zheng, Y. L., Chen, L., Liu, R. J. and *C. Y. Huang* (2007). Genetic diversity and relationship among safflower (*Carthamus tinctorius* L.) analyzed by inter-simple sequence repeats (ISSRs). *Genet. Resour. Crop Evol*. **54**, 1043-1051.

Zhang, H. L., Nagatsu, A., Watanabe, T., Sakakibara, J. and *H. Okuyama* (1997). Antioxidative compounds isolated from safflower (*Carthamus tinctorius* L.) oil cake. *Chem. Pharm. Bull*. **45**, 1910-1914.