African Journal of Biotechnology Vol. 11(91), pp. 15790-15797, 13 November, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2381
ISSN 1684–5315 ©2012 Academic Journals

*Full Length Research Paper*

# Toxicological responses in alfalfa (*Medicago sativa*) under joint stress of cadmium and napropamide

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Accepted 24 September, 2012

**Joint effects of $Cd^{2+}$ and napropamide in seeds, roots or leaves of alfalfa were investigated under different treatments. It was shown that single stress of $Cd^{2+}$ or napropamide decreased chlorophyll content after 30 days of treatment in different concentrations. The decrease in chlorophyll content became insignificant under joint stress of $Cd^{2+}$ and napropamide. It can be concluded that the interaction of $Cd^{2+}$ and napropamide would aggravate the toxic effects on chlorophyll synthesis in leaves of alfalfa. The joint effect of $Cd^{2+}$ and napropamide was markedly significant ($p < 0.05$) on the change of SP content in leaves in all treatment. Moreover, $Cd^{2+}$ and napropamide mixture exposure can increase lignin content and present synergistic effect. In a mixture treated with $Cd^{2+}$ and napropamide, 52% decrease in β-carotene content contrasted with the control in young leaves. The contents of protein thiols and non-protein thiols in the roots of alfalfa were significantly increased by $Cd^{2+}$ treatment in all treatment levels. In contrast, increasing napropamide supply did not have any significant effect on the protein thiols and non-protein thiols content. The $Cd^{2+}$ induced accumulation of $O_2^{\bullet-}$ in seeds could be increased by treatment with different $Cd^{2+}$ concentration. Production of $H_2O_2$ and $O_2^{\bullet-}$ was also higher in the napropamide treatments than in the control. The addition of napropamide significantly increased the $H_2O_2$ and $O_2^{\bullet-}$ level in the seeds of alfalfa.**

**Key words:** Alfalfa, joint stress, cadmium, napropamide.

## INTRODUCTION

The study on biochemical responses of plants to joint stress of metals and herbicides is an important area of ecotoxicology. Metals are environmental pollutants released from both industrial and agricultural sources affecting the biosphere in many places worldwide. Among them, cadmium (Cd), a nonessential element present in the atmosphere, soil, and water, is one of the most aggressive and persistent element in natural environments. Cd released into the environment may be concentrated in the soil, where it is available for the rooted plants. Due to its great solubility in water and high mobility in the soil–plant system, Cd is readily taken up by the roots (KreveŠan et al., 2003).

Agricultural soil may be contaminated with Cd as a result of industrialization, land applications of sewage sludge, and use of different fertilizers, pesticides, and insecticides (Mench, 1998; Sanita Di Toppi and Gabbrielli, 1999). The high solubility of Cd makes this element an environmental concern especially because it is easily assimilated by plants and it disturbs their metabolism (Benavides et al., 2005). Herbicide such as paraquat would also cause a significant activation of all antioxidant enzymes (Ekmekci and Terzioglu, 2005). The inhibition on protein biosynthesis is one of the popular ways in preventing plant growth by exogenous toxic chemicals. Pesticides are an indispensable controller of plant diseases and weeds for modern agriculture. Such

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pesticides get accumulated in crops or other organisms and may find their way into food chain to cause a series of secondary contaminations (Eberle and Gerber, 1976; Pylypiw et al., 1993; Nagami et al., 2004).

One of the visible toxic symptoms of the plants exposed to metals and herbicides is the change of chlorophyll content in leaves. In fact, most of the herbicides kill weeds by inhibiting their photosynthesis and then inhibiting their growth. Napropamide is a selective systemic herbicide used to limit the growth of grasses and weeds in much agricultural cultivation. When the napropamide concentration exceeds the maximum soil holding capacity, it may transfer to the surface or ground water and consequently bring contamination to aquatic or ecological systems. Commercial napropamide can easily pass into tissues of living organisms and is readily accumulated in crops and exposure of napropamide induced substantial production of $O_2^{\bullet-}$, $H_2O_2$ and oxidative injury to *Brassica napus* (Zhang et al., 2010; Biswas et al., 2007).

Alfalfa (*Medicago sativa*), a legume, is one of the most popular species used for perennial grazing and ubiquitously cultured on the global scale (Sengupta-Gopalan et al., 2007). At present, few reports are involved in the joint stress of cadmium and napropamide. However, the use of chemical fertilizers and other pesticides may introduce metals such as Cd to the terrestrial systems, which leads to combined pollution of cadmium and napropamide.

In this work, by comparable study of the biochemical responses in alfalfa under single and joint stress of cadmium and napropamide, the jointly toxicological mechanisms of cadmium and napropamide were explored. The data of this work may offer base for the assessment of ecological risks caused by joint stress of metals and organic chemicals in terrestrial ecosystems.

## MATERIALS AND METHODS

### Alfalfa culture and treatment

Seeds of alfalfa were germinated in moist filter paper for 3 days. After germination, 60 seedlings were sown in a plastic container (1 L) with 2000 g soils mixed with $Cd^{2+}$ or napropamide soil. Alfalfas were grown in a climate chamber under the controlled conditions (photoperiod, 16/8 h light/dark cycle; temperature, 20 to 25°C at day/night; relative humidity, 60%; soil moisture 60%) for 30 days (Kong et al., 2007; Cui et al., 2011). The tested concentrations for the joint stress in the present work were 0, 1.0, 5.0, and 10.0 mg/kg for $Cd^{2+}$ and 0, 5, 100 and 200 µg/kg for napropamide. There were 12 treatments with different concentration combination of $Cd^{2+}$ and napropamide and each concentration treatment had four repeats for each of the 3 days time intervals. Three repeats were performed for each of the treatments.

### Preparation of tissue extract

About 0.1 g of leaf and root tissues was ground with 1.5 ml of 50 mM pre-cooled Na-phosphate buffer (pH 7.8), containing 0.1 µM EDTA and 1% (w/v) polyvinylpyrrolidone (PVPP). The filtered tissue extract was centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was used for further analyses (Wang et al., 2009).

### Chlorophyll extract and content determination

The leaves were soaked in 80% acetone, and the chlorophyll was extracted. The extract was centrifuged at 5300 g for 10 min. Then the absorbances of the supernatant were read at 645 and 663 nm. The content of chlorophyll in alfalfa was determined in 80% acetone extract of 0.1 g leaf tissues as described by Hegedüs et al. (2001) and expressed using mg/g FW.

### Total soluble protein (SP) and lignin content measurement

The method of Bradford was used to determine the concentration of soluble proteins in leaves of alfalfa (Bradford, 1976). Absorption was recorded at 595 nm. Soluble proteins were expressed as mg/g FW. Lignin content was measured according to the method of Aline et al. (2010). Lignin was expressed as mg/g DW.

### Assay of α-tocopherol and β-carotene concentrations

The α-tocopherol was directly quantified after its extraction in order to avoid loss by oxidation with time according to the study of Emile et al. (2005). β-Carotene and chlorophyll concentrations were determined spectrophotometrically using standard curves. Sample preparation before detection was carried out as previously described by Hejazi and Wijffels (2003).

### Determination of protein thiols and non-protein thiols

The contents of total thiols were estimated according to the method of Ellman (1959). Non-protein thiols were determined following the method described by Deng and Hu (2010). The protein thiols were calculated by subtracting the non-protein thiols from total thiols.

### Determination of $H_2O_2$ and $O_2^{\bullet-}$ production

Formation of $H_2O_2$ and $O_2^{\bullet-}$ dependent on NADH peroxidase was measured colorimetrically as described by Ishida et al. (1987).

### Statistical analysis

All measurements were replicated three times. The analysis of variance (two-way ANOVA) for factors of $Cd^{2+}$ and napropamide concentrations in each time interval were performed with randomized model. The data were expressed as means ± standard error. Statistical comparisons were carried out using Origin 8.0 software, and significant differences were indicated by *letters ($p < 0.05$).

## RESULTS AND DISCUSSION

### Effects of $Cd^{2+}$ and napropamide on changes of the content of chlorophyll

Chlorophyll as a marker indicating growth status was further determined. It was shown that single stress of $Cd^{2+}$ or napropamide decreased chlorophyll content after 30 days of treatment in different concentrations. However, under joint stress of $Cd^{2+}$ and napropamide, the decrease in chlorophyll content became insignificant. That is, when

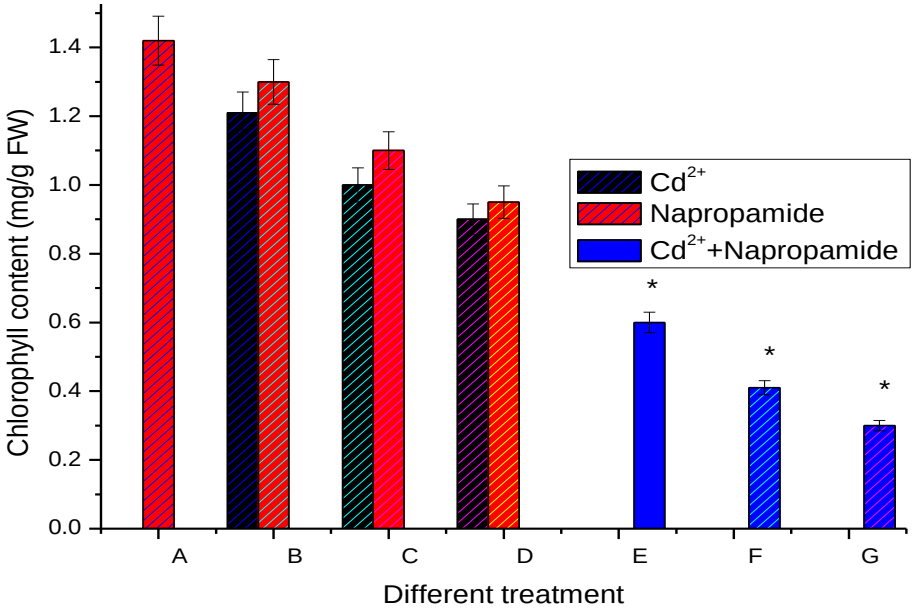


**Figure 1.** Changes of chlorophyll content under stress of single and joint stress of $Cd^{2+}$and napropamide.(A: control; B:1.0 mg/kg $Cd^{2+}$ or 5.0 µg/kg napropamide; C: 5.0 mg/kg $Cd^{2+}$ or 100 µg/kg napropamide; D: 10 mg/kg $Cd^{2+}$ or 200 µg/kg napropamide; E, F, and G: joint stress of $Cd^{2+}$and napropamide in different concentration, respectively).*$p < 0.05$.

seeds of alfalfa were treated with 1.0, 5.0, and 10.0 mg/kg $Cd^{2+}$, the contents of chlorophyll decreased by 13.16, 18.83, and 30.38% in comparison to the control, respectively. Similarly, when seeds of alfalfa were treated with napropamide at 5, 100, and 200 µg/kg, the content of chlorophyll decreased by 11.26, 15.75, and 25.69%, respectively in comparison to the control. That is, 10.0 mg/kg $Cd^{2+}$ caused the responses to be significant. For the combined treatment with 1.0 mg/kg $Cd^{2+}$ and napropamide at 5 µg/kg, the content of chlorophyll decreased in comparison to the control, the single treatment with 1.0 mg/kg $Cd^{2+}$ and the single treatment with a napropamide at 5 µg/kg.

The decrease was more evident in the other combined treatments with $Cd^{2+}$ and napropamide. The decrease in the content of chlorophyll in seeds of alfalfa treated with $Cd^{2+}$ and napropamide were higher than those of the single treatment with $Cd^{2+}$ or napropamide. In order to investigate the interaction between $Cd^{2+}$ and napropamide action on the content of chlorophyll in seeds of alfalfa, two-way ANOVA analysis was performed. ANOVA for factors of $Cd^{2+}$ and napropamide demonstrated that the joint effects of $Cd^{2+}$ and napropamide were markedly significant ($p < 0.05$) with the content of chlorophyll ($R^2 = 0.956$, $F = 3.121$, $p < 0.05$). The results indicated that there was an obvious interaction between $Cd^{2+}$ and napropamide action. Meanwhile, the degrees of decrease in toxicological effects under the combined treatment were higher than the degree in toxicological effects under the single treatment with $Cd^{2+}$ and napropamide, namely synergistic effect.

It could be concluded that the interaction of $Cd^{2+}$ and napropamide would aggravate the toxic effects on chlorophyll synthesis in leaves of alfalfa. This result is consistent with previous reports in other plants with different pesticides (Biswas et al., 2007; Song et al., 2007). Further analysis revealed that plants treated with napropamide accumulated less amounts of chlorophyll (Cui et al., 2011). Chlorophyll allows plants to obtain energy from light, which is the first stage for light reaction of photosynthesis, and thus chlorophyll is vital for photosynthesis (Wang et al., 2009; Sun and Wang, 2012). It has been reported that the single treatment with $Cd^{2+}$ or pesticides decreased the content of chlorophyll and net photosynthetic rate (Gao et al., 2010; Huang et al., 2010; Shamsi et al., 2008; Wang et al., 2009b; Zhang et al., 2010; Mahmooduzzafar et al., 2007), leading to the inhibition of the photosynthesis (Figure 1).

## Effects of $Cd^{2+}$ and napropamide on changes of SP and lignin content

Figure 2 shows the change of soluble protein (SP) content in leaves of alfalfa under single and joint stress of $Cd^{2+}$ and napropamide. The single stress of $Cd^{2+}$ had significant effect on the SP content in leaves in all the experimental exposure, and the single effect of napropamide was insignificant after 30 days of treatment. However, the joint effect of $Cd^{2+}$ and napropamide was markedly significant ($p < 0.05$) on the change of SP content in leaves in all treatment. SP content in leaves of

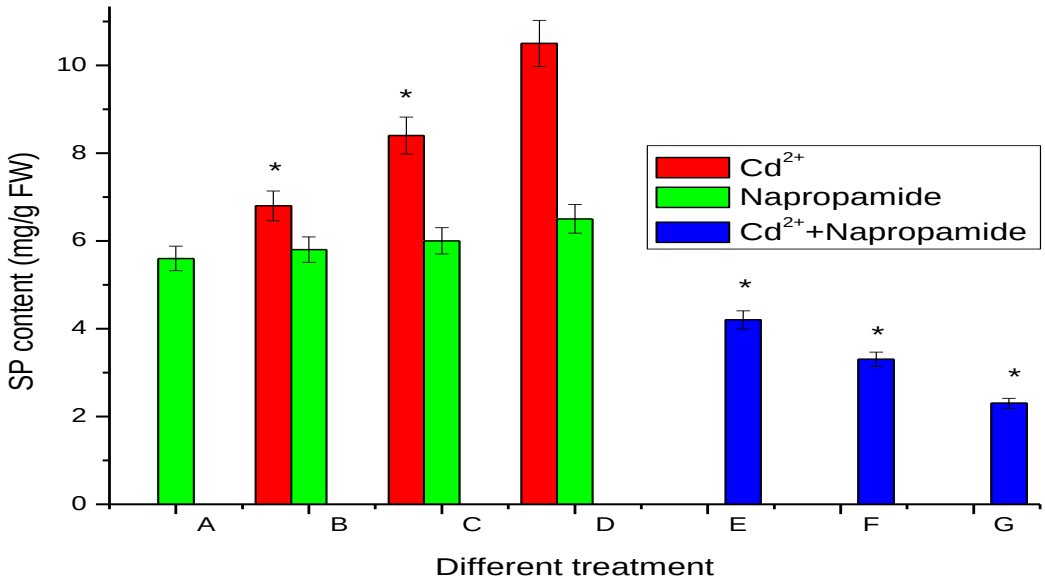


**Figure 2.** Changes of SP content under stress of single and joint stress of $Cd^{2+}$ and napropamide (A: control; B: 1.0 mg/kg $Cd^{2+}$ or 5.0 µg/kg napropamide; C: 5.0 mg/kg $Cd^{2+}$ or 100 µg/kg napropamide; D: 10 mg/kg $Cd^{2+}$ or 200 µg/kg napropamide; E, F, and G: joint stress of $Cd^{2+}$ and napropamide in different concentration, respectively). *p < 0.05.

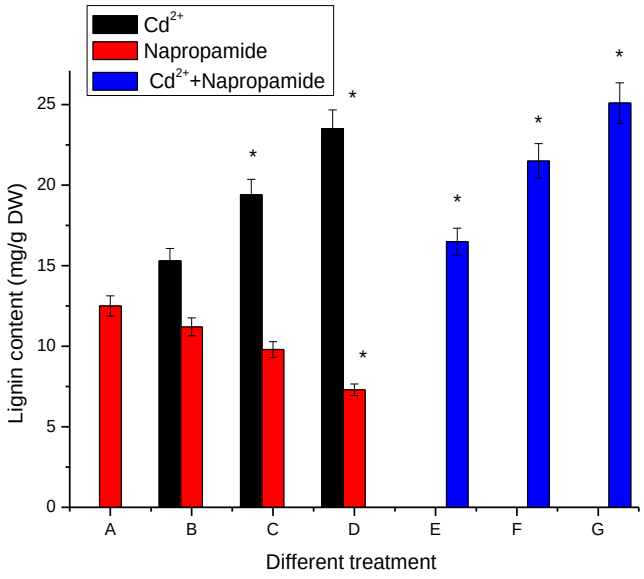


**Figure 3.** Changes of lignin content under stress of single and joint stress of $Cd^{2+}$ and napropamide (A: control; B: 1.0 mg/kg $Cd^{2+}$ or 5.0 µg/kg napropamide; C: 5.0 mg/kg $Cd^{2+}$ or 100 µg/kg napropamide; D: 10 mg/kg $Cd^{2+}$ or 200 µg/kg napropamide; E, F, and G: joint stress of $Cd^{2+}$ and napropamide in different concentration, respectively). *p < 0.05.

alfalfa increased after 30 days of treatment under single stress of $Cd^{2+}$ or napropamide, and that joint stress of $Cd^{2+}$ and napropamide significantly decreased the SP content in leaves of alfalfa compared with that under single stress of $Cd^{2+}$ or napropamide. The decrease in soluble protein (SP) content can be observed in many organisms when exposed to metals and other adverse stresses (Hegedüs et al., 2001; Jin et al., 2002).

As a consequence of $Cd^{2+}$ single exposure, lignin content increased from 12.8, 16.8 and 21.9% to 110.3, 135.6, and 167.2% after 1.0 to 10.0 mg/kg $Cd^{2+}$ treatment with respect to the control, respectively. However, lignin content decreased from 12.5, 19.7 and 25.6% to 10.3, 13.7, and 19.2% after 5.0 to 200 µg/kg napropamide single treatment with respect to the control, respectively (Figure 3). $Cd^{2+}$ and napropamide mixture exposure can increase lignin content and present synergistic effect, having ascertained that lignin content was already affected by Cd, Cu or chlorimuron-ethyl and napropamide. Moreover, the reduction in root growth has been considered one of the first effects of the Cd, Cu, Hg and herbicide (such as chlorimuron-ethyl and napropamide) associated with lignin production and related parameters (Aline et al., 2010; Zhou et al., 2008). As described earlier, the biosynthesis of lignin involves the polymerization of monolignols primarily derived from the phenylpropanoid pathway, which commences with the deamination of phenylalanine by PAL to form cinnamate, followed by the other derivatives (Song et al., 2010).

## Effects of $Cd^{2+}$ and napropamide on changes of α-tocopherol and β-carotene content

$Cd^{2+}$ toxicity affected α-tocopherol content at young leaf ages. Significant dose-related increases in α-tocopherol, and decreases in α-tocopherol was observed in the leaves of napropamide-treated alfalfas (Figure 4). After

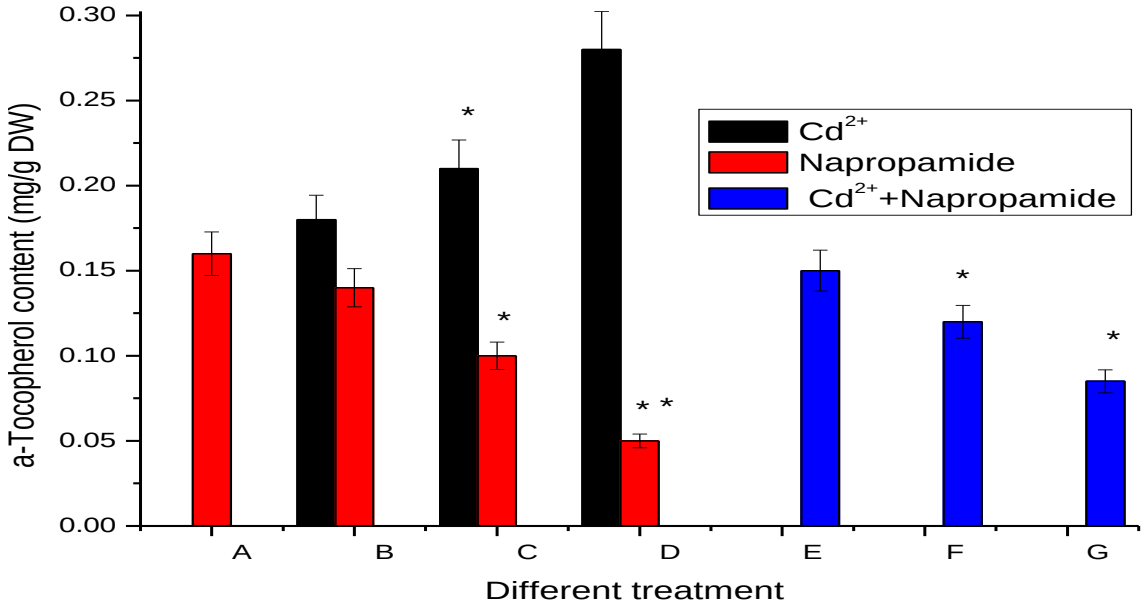


**Figure 4.** Changes of α-tocopherol content under stress of single and joint stress of $Cd^{2+}$and napropamide (A: control; B: 1.0 mg/kg $Cd^{2+}$ or 5.0 µg/kg napropamide; C: 5.0 mg/kg $Cd^{2+}$ or 100 µg/kg napropamide; D: 10 mg/kg $Cd^{2+}$ or 200 µg/kg napropamide; E, F, and G: joint stress of $Cd^{2+}$and napropamide in different concentration, respectively). *p < 0.05, **p < 0.01.

30 days of exposure to1.0, 5.0, and 10.0 mg/kg $Cd^{2+}$, a 21, 35, and 48% raise in α-tocopherol content was detected compared to the control in young leaves of alfalfa, respectively. In an opposite way, the α-tocopherol content was reduced at both napropamide treatments. At 5.0 µg/kg napropamide supply, α-tocopherol content declined in young leaves by 25% as compared to the control. Whereas, the α-tocopherol content decreased significantly at 200 µg/kg napropamide exposure. Further, mixture treated with joint stress of $Cd^{2+}$ (10.0 mg/kg) and napropamide (200 µg/kg), the 45% decrease in α-tocopherol content contrasted with the control in young leaves. Metabolites such as α-tocopherol is also involved in antioxidant defense. α-Tocopherol is the major vitamin E compound found in leaf chloroplasts (Munné-Bosch, 2005). This antioxidant deactivates photosynthesis-derived reactive oxygen species (ROS) and prevents the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes (Munné-Bosch, 2005). Under $Cd^{2+}$ stress, it has been shown that high α-tocopherol content plays a major role in plant stress tolerance, keeping an adequate redox state in chloroplasts, while decreased levels facilitate oxidative damage (Munné-Bosch, 2005).

$Cd^{2+}$ treatment induced changes of β-carotene in the young leaves depending on the $Cd^{2+}$ concentration (Figure 5). The β-carotene contents of young leaves treated with 1.0 mg/kg $Cd^{2+}$ did not show remarkable variations as compared to the control. In contrast, 10.0 mg/kg $Cd^{2+}$ treatments resulted in 38% reduction of total β-carotene content. In young leaves, β-carotene contents were significantly affected by napropamide treatments at any concentration. Mixture treated with joint stress of $Cd^{2+}$ and napropamide, the 52% decrease in β-carotene content contrasted with the control in young leaves at the maximum mixture concentration. β-Carotene, a tetraterpenoid containing eight isoprene units is a known precursor of vitamin A and accounts for more than 90% of total carotenoids in vegetables, β-carotene not only serves as valuable source of vitamin A, but also serves as a potent antioxidant, scavenging free radicals and quenching singlet oxygen (Rosa and Marta, 2003).

Our results indicate that β-carotene content was reduced in young leaves at $Cd^{2+}$ and napropamide with a decrease of both chlorophyll and β-carotene contents. $Cd^{2+}$ and napropamide concentrations affect the electron transport rates of photo system I (PSI) and photo system II (PSII), therefore generating high level of free oxygen radicals (Herbette et al., 2006; Ekmeki et al., 2008; Cui et al., 2011), it is likely that the reduction in chlorophyll content is a direct consequence of the reduction of β-carotene and β-carotenoid accumulation which in turn limits the ROS detoxification capacity.

### Effects of $Cd^{2+}$ and napropamide on changes of protein thiol and non-protein thiol contents

Protein thiols and non-protein thiol compounds can be found in most plants, micro-organisms and all mammalian tissues. The contents of protein thiols and non-protein thiols increased in the roots of alfalfa exposed to $Cd^{2+}$ treatment compared with the control group (Figure 6A and B) and the napropamide exposures were insigni-

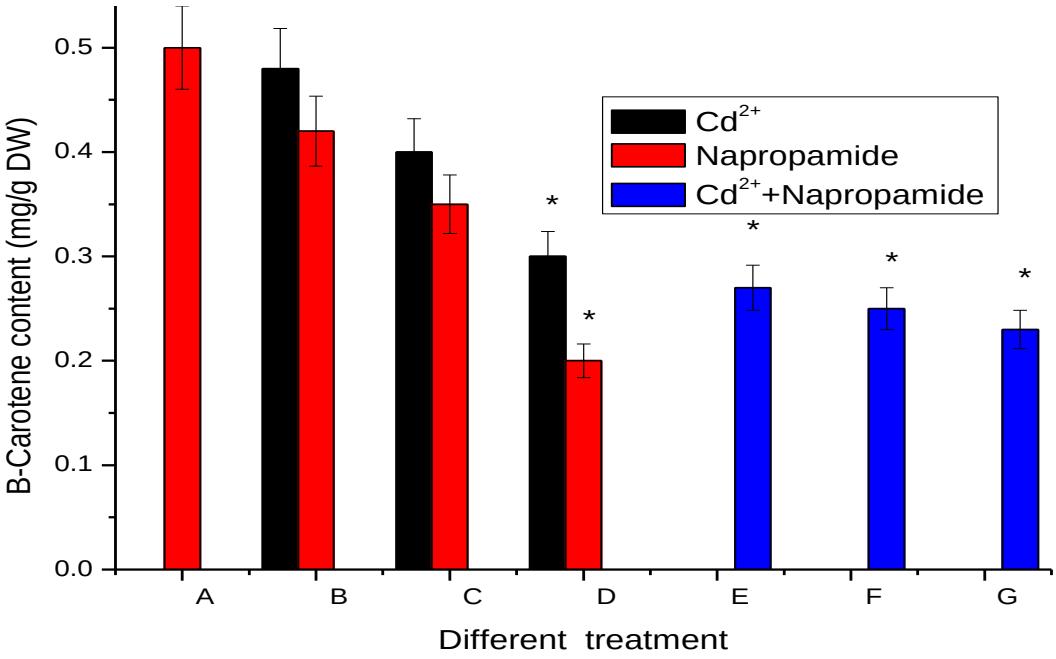


**Figure 5.** Changes of β-carotene content under stress of single and joint stress of $Cd^{2+}$and napropamide (A: control; B: 1.0 mg/kg $Cd^{2+}$ or 5.0 µg/kg napropamide; C: 5.0 mg/kg $Cd^{2+}$ or 100 µg/kg napropamide; D: 10 mg/kg $Cd^{2+}$ or 200 µg/kg napropamide; E, F, and G: joint stress of $Cd^{2+}$and napropamide in different concentration, respectively). *p < 0.05.

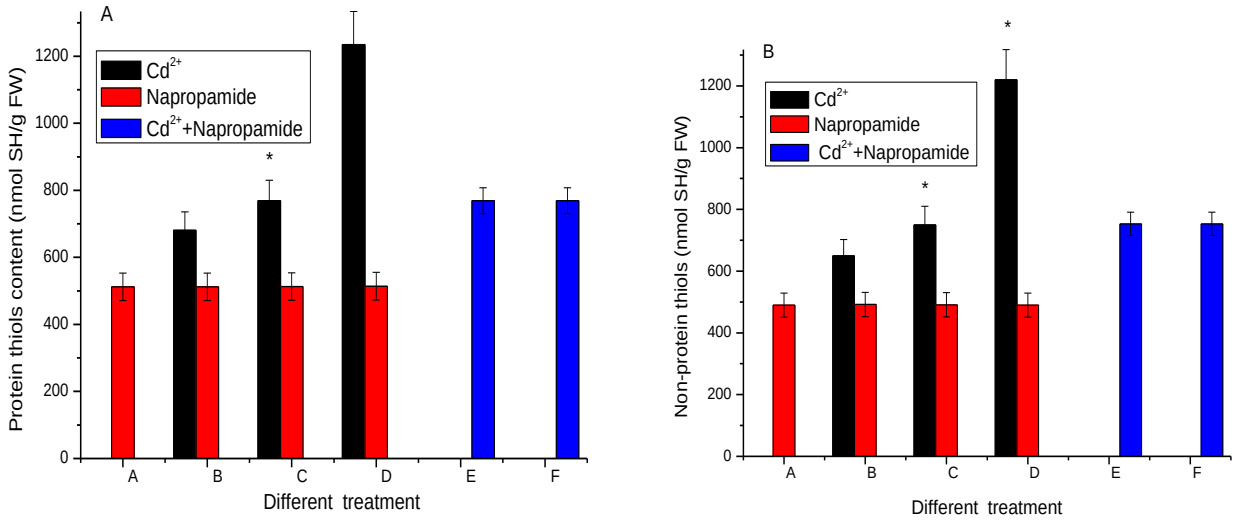


**Figure 6.** Changes of protein thiol (A) and non-protein thiol; (B) content under stress of single and joint stress of $Cd^{2+}$and napropamide. (A: control; B: 1.0 mg/kg $Cd^{2+}$ or 5.0 µg/kg napropamide; C: 5.0 mg/kg $Cd^{2+}$ or 100 µg/kg napropamide; D: 10 mg/kg $Cd^{2+}$ or 200 µg/kg napropamide; E: 5.0 mg/kg $Cd^{2+}$ and 100 µg/kg napropamide; F: 5.0 mg/kg $Cd^{2+}$ and 200 µg/kg napropamide). *$p$ < 0.05.

ficant increase in the contents of protein thiols and non-protein thiols in the roots of alfalfa. The contents of protein thiols and non-protein thiols in the roots of alfalfa were significantly increased by $Cd^{2+}$ treatment in all levels treatment. The highest contents of protein thiols and non-protein thiols in alfalfa were observed that received 10 mg/kg $Cd^{2+}$, value was 123 and 115% greater than that of the control, respectively. In contrast, increasing napropamide supply did not have any significant effect on the protein thiols and non-protein thiols content. The influence of protein thiols and non-protein thiol upon metals is due to their extremely high affinity for -SH residues (Deng

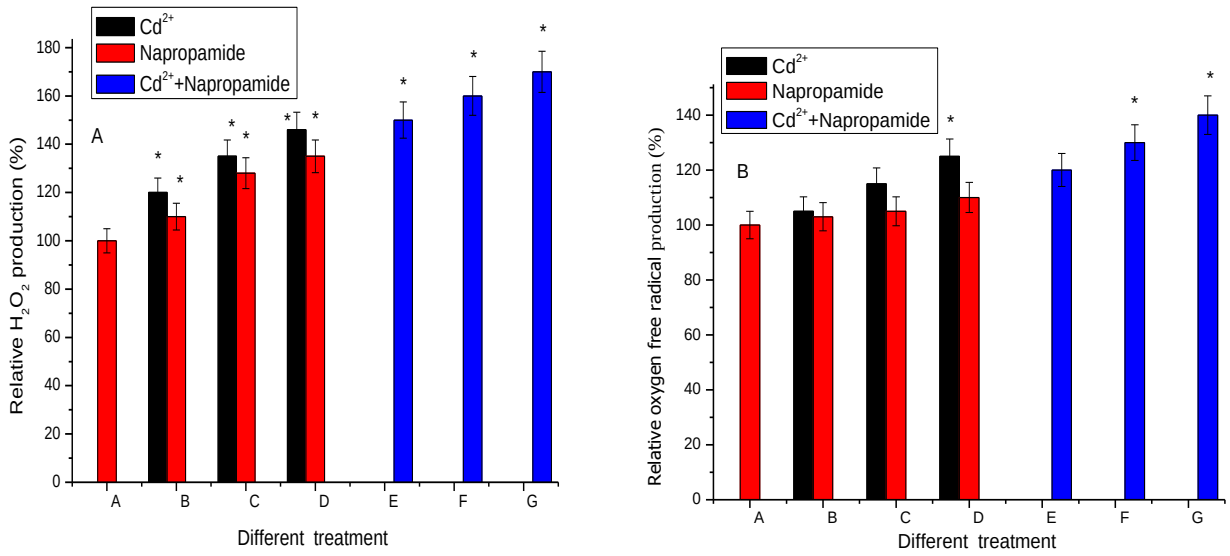


**Figure 7.** Changes of $H_2O_2$ (A) and $O_2^{\bullet-}$(B) level under stress of single and joint stress of $Cd^{2+}$and napropamide. (A: control; B: 1.0 mg/kg $Cd^{2+}$ or 5.0 µg/kg napropamide; C: 5.0 mg/kg $Cd^{2+}$ or 100 µg/kg napropamide; D: 10 mg/kg $Cd^{2+}$ or 200 µg/kg napropamide; E, F, and G: joint stress of $Cd^{2+}$ and napropamide in different concentration, respectively). *$p$ < 0.05.

and Hu, 2010).

### Effects of $Cd^{2+}$ and napropamide on changes of $H_2O_2$ and $O_2^{\bullet-}$ production

Changes of NADH-dependent $H_2O_2$ production in germinating seeds of alfalfa are shown in Figure 7A and B. The $H_2O_2$ content was about 120, 135, and 146% of the control value, and it was significantly higher than that of the control after 30 days in the 1.0, 5.0, and 10.0 mg/kg $Cd^{2+}$ treated seeds, respectively. Similarly, the $Cd^{2+}$ induced accumulation of $O_2^{\bullet-}$ in seeds could be increased by treatment with different $Cd^{2+}$ concentration. Production of $H_2O_2$ and $O_2^{\bullet-}$ was also higher in the napropamide treatments than in the control with different treatments. The addition of napropamide significantly increased the $H_2O_2$ level in the seeds of alfalfa. Treated with napropamide induced high level of $O_2^{\bullet-}$ in seeds of alfalfa compared with the control, indicating that a higher level of $O_2^{\bullet-}$ was produced with different napropamide concentration. The seeds of alfalfa were treated with joint stress of $Cd^{2+}$ and napropamide, the results showed that $H_2O_2$ and $O_2^{\bullet-}$ accumulation in seeds of alfalfa significantly increased under treatment with different joint stress of $Cd^{2+}$ and napropamide. Protective effects of exogenous $H_2O_2$ against abiotic stresses were observed in plant seed germination and seedling growth (He et al., 2009; Singh et al., 2004). $O_2^{\bullet-}$ represents an instable species of reactive oxygen which can rapidly be converted to $H_2O_2$, and therefore, production of $O_2^{\bullet-}$ by plants is usually accompanied by the appearance of $H_2O_2$ (Zhou et al., 2008; Cui and Hong, 2010; Elbaz et al., 2010). The sensitive generation of $H_2O_2$ and $O_2^{\bullet-}$ can be used as a biomarker to illustrate the degree of oxidative stress.

## Conclusion

The significantly joint effect of $Cd^{2+}$ and napropamide on alfalfa at biochemical levels was prevalent in the natural environment. It was shown that single stress of $Cd^{2+}$ or napropamide decreased chlorophyll content after 30 days of treatment in different concentrations. The decrease in chlorophyll content became insignificant under joint stress of $Cd^{2+}$ and napropamide. It could be concluded that the interaction of $Cd^{2+}$ and napropamide would aggravate the toxic effects on chlorophyll synthesis in leaves of alfalfa. The joint effect of $Cd^{2+}$ and napropamide was markedly significant ($p < 0.05$) on the change of SP content in leaves in all treatment. Moreover, $Cd^{2+}$ and napropamide mixture exposure can increase lignin content and present synergistic effect on mixture treated with joint stress of $Cd^{2+}$ and napropamide; the 45% decrease in α-tocopherol content contrasted with the control in young leaves.

In mixture treated with joint stress of $Cd^{2+}$ and napropamide, the 52% decrease in β-carotene content contrasted with the control in young leaves. The contents of protein thiols and non-protein thiols in the roots of alfalfa were significantly increased by $Cd^{2+}$ treatment in all treatment levels. In contrast, increasing napropamide supply did not have any significant effect on the protein thiols and non-protein thiols content. The $Cd^{2+}$ induced accumulation of $O_2^{\bullet-}$ in seeds could be increased by treatment with different $Cd^{2+}$ concentration. Production of $H_2O_2$ and $O_2^{\bullet-}$ was also higher in the napropamide treatments than in the control. The addition of napropamide significantly increased the $H_2O_2$ level in the seeds of alfalfa. Treated with napropamide induced high level $O_2^{\bullet-}$ in seeds of alfalfa compared with the control, indicating

that a higher level of $O_2^{\bullet-}$ was produced with different napropamide concentration and the sensitive generation of $H_2O_2$ and $O_2^{\bullet-}$ can be used as a biomarker to illustrate the degree of oxidative stress.

## ACKNOWLEDGEMENTS

This research was supported by Chongqing Key Laboratory of Micro/Nano Materials Engineering and Technology (No. KFJJ1106), Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection (No.JLCBE11004), and Jiangsu Marine Resources Development Research Institute (No. JSIMR10E04).

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