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Accumulation and residue of napropamide in alfalfa (*Medicago sativa*) and soil involved in toxic response

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ABSTRACT

Napropamide belongs to the amide herbicide family and widely used to control weeds in farmland. Intensive use of the herbicide has resulted in widespread contamination to ecosystems. The present study demonstrated an analysis on accumulation of the toxic pesticide napropamide in six genotypes of alfalfa (*Medicago sativa*), along with biological parameters and its residues in soils. Soil was treated with napropamide at 3 mg kg^{-1} dry soil and alfalfa plants were cultured for 10 or 30 d, respectively. The maximum value for napropamide accumulation is 0.426 mg kg^{-1} in shoots and 2.444 mg kg^{-1} in roots. The napropamide-contaminated soil with alfalfa cultivation had much lower napropamide concentrations than the control (soil without alfalfa cultivation). Also, the content of napropamide residue in the rhizosphere was significantly lower than that in the non-rhizosphere soil. *M. sativa* exposed to 3 mg kg^{-1} napropamide showed inhibited growth. Further analysis revealed that plants treated with napropamide accumulated more reactive oxygen species (O_2^- and H_2O_2) and less amounts of chlorophyll. However, not all cultivars showed oxidative injury, suggesting that the alfalfa cultivars display different tolerance to napropamide.

1. Introduction

Pesticides are an indispensable controller of plant diseases and weeds for modern agriculture. Application of pesticides ensures not only the stable production but also the quality of crops to sustain the growing world population [1]. Since a large number of active ingredients are incorporated into pesticides, some of them are persistent in soils for many years [2]. Such pesticides get accumulated in crops or other organisms and may find their way into food chain to cause a series of secondary contaminations [3–5]. The removal of toxic pesticides using plants that has potential to accumulate and degradate the organic xenobiotics has been accepted an advantageous approach over other commonly used methods in costs and practice [6]. These tolerant plants for potential phytoremediation or phytodegradation purpose can be used for scavenging the toxic compound from the soil.

Napropamide [*N*,*N*-diethyl-2-(1-naphthalenyloxy) propanamide] is a selective systemic herbicide used to limit the growth of grasses and weeds in many agricultural cultivations [7]. The half-life of napropamide is approximate 70 d, provided it sneaks into the soil layer [8]. Commercial napropamide can easily pass into tissues of living organisms and is readily accumulated in crops [9]. For example, the residue of napropamide in tea leaves was detectable in the first several days after its application [10]. 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.

Import of pesticides to plants disrupts biochemical/physiological metabolisms [4,11]. Exposure of napropamide induced substantial production of $O_2^{-\bullet}$, H_2O_2 and oxidative injury to *Brassica napus* [10]. However, some plants tolerant to organic xenobiotics can remove pesticides by taking them up from environment via roots and making them degraded via putative metabolic pathway [6,11]. But, such efficient plants used for phytodegradation are very limited. Genotype screen and identification of plant species are the first step to meet the requirement. Alfalfa (*Medicago sativa*), a legume, is one of the most popular species used for perennial grazing and ubiquitously cultured on the global scale [12]. Most ecotypes of alfalfa grow under adverse environmental conditions than other perennial species. These diverse ecotypes are adaptive to various environmental stresses such as cold, hot and dry climates or even organic and inorganic polluted soils [13]. Recent studies have demonstrated that alfalfa has a higher

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tolerance to toxic heavy metals than many other plant species and some of them can remove metal ions from contaminated soil and aqueous solution [14–16]. However, little is known about the potential of alfalfa to accumulate or degrade organic xenobiotics in plants and soils. In view of the environmental relevance of the pesticide contamination, alfalfa was used to investigate the plant accumulation and residue of napropamide in the pesticide-contaminated soil. We comparatively analyzed the characteristics of napropamide accumulation and biological response of the plant to napropamide with the several cultivars. Thus, the objective of this study was to evaluate the ability of alfalfa to tolerate the pesticide and seek a set of sensitive biomarkers for diagnosing potential effects of napropamide on plants. The study will help our understanding of accumulative and tolerance mechanisms in the plant species, develop an appropriate method that can be used to estimate the degree of toxicity in the herbicide-contaminated soil and evaluate the potential phytoremediation of alfalfa to napropamide-contaminated soil.

2. Materials and methods

2.1. Materials

Napropamide-free soil (pH 7.65; organic matter, 1.40%; total N, 1.26 g kg^{-1}; available P 34.3 mg kg^{-1}; and available K, 91.5 mg kg^{-1}) was collected from the Experimental Station of Nanjing Agricultural University, Nanjing, China (Eutric gleysols, N 32.03°; E 118.84°). Napropamide (98% purity) was obtained from Rudong Pesticide Institute, Jiangsu, China. Seeds of six genotypes of alfalfa (*Medicago stativa*, cv. Golden Empress, Zhong Mu 1, WL232, WL323, Gannong 3 and Longdong) were tested in this study.

2.2. Plant culture and treatment

Seeds of alfalfa were germinated in moist filter paper for 1 d. After germination, seedlings (20) were sown in a plastic container (1 L) with 1120 g soils mixed with napropamide at 3 mg kg^{-1} soil. Plants were grown in a climate chamber under the controlled conditions (light intensity, 400 μmol m^{-2} s^{-1}; photoperiod, 16/8 h light/dark cycle; temperature, 25/21 °C at day/night; relative humidity, 60%; soil moisture 60%) for 10 or 30 d [17]. After treatment, the plant roots and shoots were separately harvested.

When napropamide in rhizosphere was analyzed, plants were removed carefully from the soil. Rhizosphere soil (adhering to the root system) was collected by carefully taking out the plants from the soil and shaking the root system to remove clumps of soils adhering to the roots [18,19]. The soil unadhered to the root system was collected as non-rhizosphere soil.

2.3. Analysis of napropamide

Fresh shoot (2 g) of plants (20 seedlings) was ground with 15 mL acetone–water (3:1, *V*:*V*) and ultra-sonicated for 30 min, respectively. The mixture was filtrated and the filtrate was vaporized to remove acetone at 30 °C in a rotary vacuum evaporator. Water in the mixture was extracted by dichloromethane for three times, each with 10 mL dichloromethane. The water phase was removed and the dichloromethane phase was concentrated to dryness at 30 °C. The residue was dissolved in 3 mL petroleum ether and passed through a florisil solid phase extraction (SPE) column. Elutes were discarded. The column was washed with 10 mL petroleum ether:acetone (4:1, *V*:*V*). The washing solution was collected and concentrated to dryness at 40 °C in a rotary vacuum evaporator. The residue was dissolved in 1 mL methanol for high performance liquid chromatography (HPLC) analysis. Extraction, purification and concentration procedures for alfalfa roots were performed as described above. Alfalfa tissues were oven-dried at 70 °C for 72 h and weighed. Calculation of napropamide content was based on the dry weight.

Napropamide in the soil were determined following the method described by Guo et al. [20], with slight modifications. Soil (20 g) was extracted with 40 mL acetone–water (3:1, *V*:*V*) in an ultrasonic bath for 30 min. The extracting solution was centrifuged at 6000 *g* for 5 min and filtered. The sample was re-extracted with 15 mL acetone–water (3:1, *V*:*V*), centrifuged and filtered. The filtrate was concentrated to remove acetone by rotary vacuum evaporator at 30 °C. The residual water was extracted by petroleum ether for three times, each time with 10 mL petroleum ether. The organic phase was collected and evaporated to dryness. The residue was dissolved in 1 mL methanol for HPLC analysis. Napropamide from shoot, root and soil was determined by HPLC [20].

2.4. Determination of metabolites

Chlorophyll extraction and quantification were performed by homogenizing 0.1 g fresh leaves in 8 mL of 80% acetone (pH 7.8), followed by centrifugation at 5000 *g* for 10 min. The supernatant was subject to spectrophotometrical assay and total chlorophyll was calculated [21]. Accumulation of lipid peroxides (thiobarbituric acid reactive substances, TBARS) in tissues were determined and expressed as equivalent of malondialdehyde based on the method described previously [22].

2.5. Histochemical detection of O_2^- and H_2O_2

Superoxide (O_2^-) was determined using nitroblue tetrazolium (NBT) as a substrate [23]. Leaves were excised at the base of stem and supplied with 10 mM Na-citrate buffer (pH 6.0) containing 6 mM NBT under light at 25 °C for 8 h. The leaves were decolorized in boiling ethanol (95%) for 10 min. The deep blue product (reaction of NBT with O_2^-) was visualized.

The cellular hydrogen peroxide (H_2O_2) was determined using 3,3-diaminobenzidine (DAB) as a substrate [23]. Leaves were loaded through the cut stems with a 1 mg mL^{-1} solution of DAB (pH 3.8) under light at 25 °C for 8 h. The leaves were decolorized in boiling 95% ethanol for 10 min. The deep brown polymerization product (reaction of DAB with H_2O_2) was visualized.

2.6. Statistical analysis

All experiments in the study were independently performed three times. The data shown in figures and tables were the mean of three replicates of treatments (means ± SD). Significance of differences between the treatments was statistically evaluated by standard deviation and Duncan's test methods ($p < 0.05$).

3. Results

3.1. Accumulation and translocation of napropamide in alfalfa

The maximum napropamide-accumulating cultivar was WL232, in which the napropamide concentration was 2.444 mg kg^{-1} FW in roots (Table 1). Gannong 3, Longdong and Zhong Mu 1, had contents of 1.723, 1.472 and 1.133 mg kg^{-1} FW in roots, respectively. The cultivars that had lower levels of napropamide were Golden Empress and WL323, and each of them had the napropamide contents of 0.865 and 0.651 mg kg^{-1} FW, respectively. Amongst the six cultivars, the pattern of napropamide accumulation in roots was not always consistent with that of shoots. The contents of napropamide in shoots were in the declining order: Golden Empress > Longdong > Gannong 3 > WL232 > WL323 > Zhong Mu 1. In any cultivars, the shoots always accumulated less napropamide

Table 1
Napropamide accumulation in soils and alfalfa. Plants: Golden Empress, Zhong Mu 1, WL232, WL323, Longdong and Gannong 3. The bioconcentration factors (BCF) and translocation factor (TF) for napropamide in plants were analyzed. Values are the means ± SD.

Plants	Root (mg kg^{-1} FW)	Shoot (mg kg^{-1} FW)	Root BCF[a]	Shoot BCF[a]	TF[b]	Soil (mg kg^{-1} dry soil)
Control	–	–	–	–	–	1.784 ± 0.096a
Golden Empress	0.865 ± 0.050d	0.426 ± 0.049a	0.700	0.345	0.493	1.235 ± 0.092b
Zhong Mu 1	1.133 ± 0.237d	0.111 ± 0.075c	1.776	0.174	0.098	0.638 ± 0.135c
WL232	2.444 ± 0.039a	0.280 ± 0.012b	2.526	0.289	0.115	0.968 ± 0.091b
WL323	0.651 ± 0.042e	0.208 ± 0.043c	0.834	0.267	0.320	0.780 ± 0.025c
Gannong 3	1.723 ± 0.062b	0.298 ± 0.010b	1.553	0.269	0.173	1.109 ± 0.047b
Longdong	1.472 ± 0.042c	0.337 ± 0.050a	1.411	0.323	0.229	1.043 ± 0.035b

Means followed by different letters are significantly different between genotypes ($p < 0.05$).
[a] BCF: fresh weight ratio of napropamide concentration in plant to the soil.
[b] TF: ratio of shoot BCF to root BCF; and FW: fresh weight.

than the roots. This may be the result that the root was in direct contact with napropamide, whereas the shoot accumulation of napropamide needed translocation from root.

We further investigated long-term exposure to napropamide using Zhong Mu 1. When the plant was cultured in soil containing 3 mg kg^{-1} napropamide for 30 d, the levels of napropamide in roots and shoots were 0.312 and 0.152 mg kg^{-1} FW, respectively (Table 2). Compared with the result of 10 d, the 30 d exposure resulted in increased concentration of napropamide in shoots but reduced in roots, suggesting that a proportion of root-accumulated napropamide was translocated into the above-ground. Alternatively, the root napropamide might be degraded by the metabolic systems when the plant underwent the long term exposure to napropamide.

To get insights into the genotype differences among the plants, we analyzed bioconcentration factor (BCF) and translocation factor (TF) for alfalfa cultivars under napropamide exposure. The BCF refers to the quotient between the organism and medium substance concentrations [24]. The BCF values for roots changed considerably, with the BCF values ranging from 0.700 to 2.526. WL232 was a dominant cultivar in uptake of napropamide into roots, whereas Golden Empress had the least (Table 1). Compared with the root BCF values, those for shoots showed less variation. Both Golden Empress and Longdong showed the highest BCF value, implying that more napropamide was transported to the above-ground from soil. Translocation factor (TF) reflects the ratio of napropamide concentration in shoot to root and can be used to evaluate the plant capability of translocating napropamide from root to shoot. The TF value was greater for Golden Empress than that of the other cultivars, indicating that napropamide moved more rapidly in this cultivar. By contrast, Zhong Mu 1 had lower TF, indicating the slower movement from root to shoot (Table 1).

3.2. Napropamide residue in soil

Rhizospheric napropamide was determined using 10 d long alfalfa-planted soils. The content of napropamide in the non-rhizospheric soil was higher than that in the rhizospheric soil for all cultivars. Relative to its control, Zhong Mu 1 showed the lowest abundance of napropamide in the rhizosphere, with only 33.0% of the control (Fig. 1). The residues for the other cultivar-planted rhizosphere ranged from 35.9% to 80.0% of the control. We also performed a long-term experiment (30 d) with Zhong Mu 1. The similar tendency in both rhizospheric and non-rhizospheric soils was observed (Table 2). Following exposure for 30 d, the content of napropamide in Zhong Mu 1 rhizosphere was 43.7% of that in the non-rhizospheric soil. However, the residue of napropamide in rhizosphere was much less than that in 10 d alfalfa-planted soils (Fig. 1).

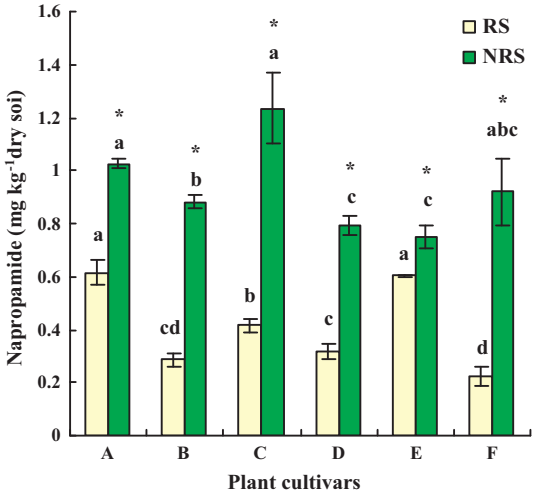

Fig. 1. Napropamide concentration in rhizospheric (RS) and non-rhizospheric (NRS) soils with *Medicago sativa* planted for 10 d. Plants: A, Golden Empress; B, Zhong Mu 1; C, WL232; D, WL323; E, Gannong 3; F, Longdong. Values are the means ± SD ($n = 3$). Asterisks indicate that mean values are significantly different between the RS and NRS treatments ($P < 0.05$). Means followed by different letters are significantly different between genotypes ($p < 0.05$).

3.3. Effect of napropamide on growth and chlorophyll

Root elongation of alfalfa plants was significantly inhibited (Fig. 2). Compared with the control, the root elongation of Golden Empress, Zhong Mu 1, WL232, WL323, Gannong 3 and Longdong was decreased by 74.2%, 79.5%, 77.3%, 72.8%, 64.9% and 68.7%, respectively (Fig. 2a). It is apparent that roots were sensitive to napropamide. Determination of biomass revealed that the dry weight of alfalfa was also affected by napropamide exposure (Fig. 2b). WL232 was least affected, whereas Golden Empress was most affected among the six by napropamide exposure (Fig. 2b).

Chlorophyll as a marker indicating growth status was further determined. The chlorophyll levels in leaves with 3 mg kg^{-1} napropamide were consistently lower than those of the controls (Fig. 3). As a sensitive cultivar, Golden Empress showed the most reduction in chlorophyll contents, which was decreased to 65.5% of the control. The chlorophyll contents in the other alfalfa plants were changed differently.

3.4. Effect of napropamide on H_2O_2 and O_2^- generation and peroxidation

Histochemical staining of O_2^- with nitroblue tetrazolium (NBT) showed that treatment with napropamide induced intense staining of O_2^- in leaves compared with the control (Fig. 4a), indicating

Table 2
Napropamide accumulation in soil and alfalfa (Zhong Mu 1).The control soil also contained 3 mg kg^{-1} napropamide, but no alfalfa was planted on it. After cultivation, the plants were harvested and napropamide was quantified. Values are the means ± SD. Means followed by different letters are significantly different between the soils with plants and without plants (Control) ($p < 0.05$).

Plants	Napropamide concentration				
	Root (mg kg^{-1} FW)	Shoot (mg kg^{-1} FW)	Rhizosphere (mg kg^{-1} dry soil)	Non-rhizosphere (mg kg^{-1} dry soil)	Soil (mg kg^{-1} dry soil)
Control	–	–	–	–	1.005 ± 0.091a
Zhong Mu 1	0.312 ± 0.018	0.152 ± 0.016	0.101 ± 0.029	0.231 ± 0.114	0.227 ± 0.052b

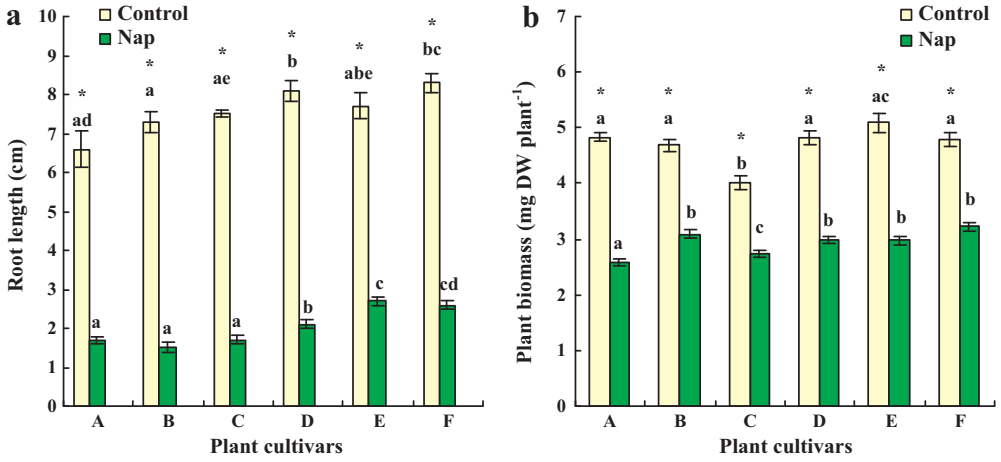

Fig. 2. Effect of napropamide on the growth of *Medicago sativa*. Control: napropamide-free. Plants: A, Golden Empress; B, Zhong Mu 1; C, WL232; D, WL323; E, Gannong 3; F, Longdong. Values are the means ± SD ($n = 3$). Asterisks indicate the significant differences between the treatments and the control ($P < 0.05$). Means followed by different letters are significantly different between genotypes ($p < 0.05$).

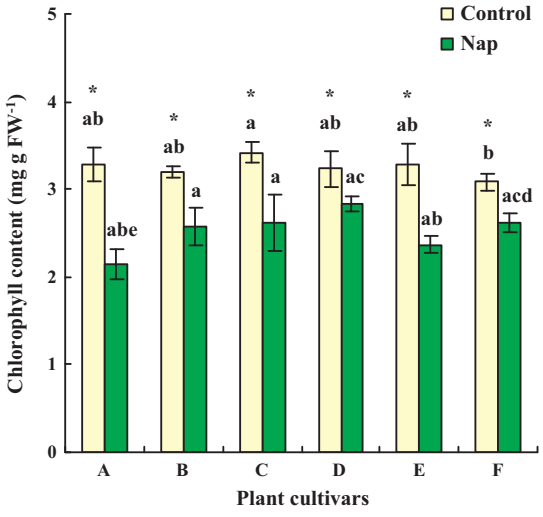

Fig. 3. Effect of napropamide on accumulation of chlorophyll in the leaves of *Medicago sativa*. Control: napropamide-free. Plants: A, Golden Empress; B, Zhong Mu 1; C, WL232; D, WL323; E, Gannong 3; F, Longdong. Values are the means ± SD ($n = 3$). Asterisks indicate the significant differences between the treatments and the control ($P < 0.05$). Means followed by different letters are significantly different between genotypes ($p < 0.05$).

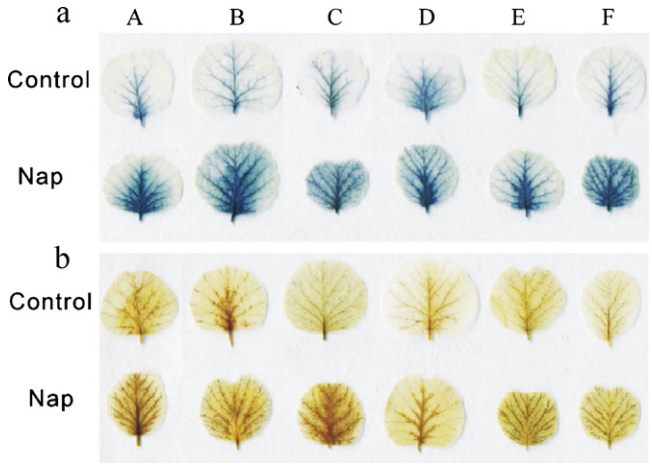

Fig. 4. Effect of napropamide on generation of superoxide radical (a) and hydrogen peroxidase (b) in *Medicago sativa*. Plants: A, Golden Empress; B, Zhong Mu 1; C, WL232; D, WL323; E, Gannong 3; F, Longdong.

that a higher level of O_2^- was produced. Hydrogen peroxidase (H_2O_2), is another important ROS and was visualized in leaves using 3,3-diaminobenzidine (DAB) as a substrate (Fig. 4b). When napropamide was accumulated in the leaves, they were also stained extensively.

The content of lipid peroxides was generally higher in napropamide-treated plants than those in the control (Fig. 5). However, three cultivars (B, Zhong Mu 1, E, Gannong 3 and F, Longdong) had significantly higher contents in leaves compared with the control. In roots, only Zhong Mu 1 (B) and WL232 (C) was found to have significantly higher levels of TBARS.

4. Discussion

Alfalfa plants can accumulate napropamide but the capability of accumulation varied considerably. Simultaneously, soil cultivation with alfalfa showed significantly low residues of napropamide in rhizosphere. Such uptake and/or degradation of napropamide resulted in the removal of napropamide from the rhizospheric soil. These results indicate that *M. sativa* is useful and can be designed to improve the tolerance to and accumulate the herbicide in the environment.

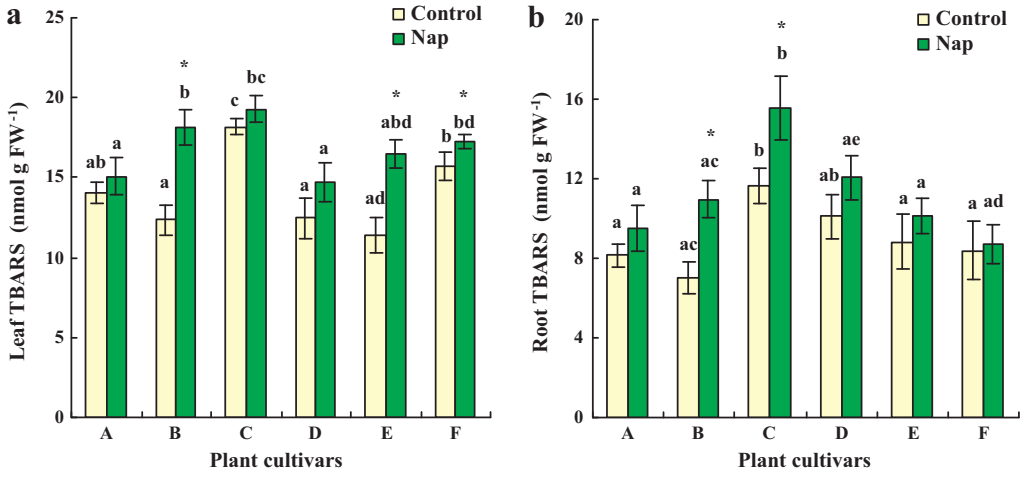

Fig. 5. Effect of napropamide on accumulation of TBARS in leaves (a) and roots (b) of *Medicago sativa*. Control: napropamide-free. Plants: A, Golden Empress; B, Zhong Mu 1; C, WL232; D, WL323; E, Gannong 3; F, Longdong. Values are the means ± SD ($n = 3$). Asterisks indicate the significant differences between the treatments and the control ($P < 0.05$). Means followed by different letters are significantly different between genotypes ($p < 0.05$).

Our analysis revealed that the six cultivars varied in its uptake of napropamide and subsequent translocation from root to shoot. WL232 was the cultivar that accumulated the higher amounts of napropamide in root and Golden Empress was the most efficient accumulator in shoot. Compared with the shoot, the root accumulated more herbicides in the plants. This result is consistent with previous reports in other plants with different pesticides [10,25]. Although some cultivars accumulated abundantly napropamide in the root, the root-loaded napropamide could not be properly translocated into the above-ground. For instance, WL232 was the efficient accumulator with the highest concentration of napropamide (2.444 mg kg^{-1} FW) in roots, but its translocation from root to shoot was rather weak (TF, 0.115). By contrast, Golden Empress accumulated less quantity of napropamide in the root but more napropamide was present in the shoot. The uptake of pesticides by plants depends on plant species, chemical properties of the compounds, way of application, soil type and environmental conditions [10,26,27]. This result indicates that plant genotype is one of the major determinants for accumulation of the pesticide.

The levels of napropamide residues in the control soil (without plants) were higher than those with alfalfa cultivated. This suggests that all cultivars had a contribution not only to accumulation of the herbicide but as well to the degradation of napropamide in the soil. Amongst the soils in which alfalfa was grown, the soil with Zhong Mu 1 had the lowest level of napropamide residues in soil (0.638 mg kg^{-1} dry soil), suggesting that the cultivar has the greater ability to facilitate plant accumulation or decay of napropamide in the soil. The concentration and persistence of pesticides in environment depend on several factors including crops planted, soil type, microbial-mediated degradation and environmental conditions [28]. Of these, microbial degradation is considered as one of the dominant approaches leading to the efficient degradation. The microbial population in the rhizosphere is usually higher than those in the non-rhizosphere. Exudates from plant roots provide nutrition to rhizosphere microbes and stimulate microbiological activity in the rhizosphere soil [19,29,30]. Organic pollutants are most likely to be degraded in the rhizosphere either by exuded enzymes or by the enhanced microbial community [31]. In this study, the residues of napropamide in the rhizosphere with alfalfa were significantly lower than those in non-rhizosphere, indicating that cultivation of alfalfa enhanced the napropamide degradation in the soil. Also, the release of root exudates might promote the plant uptake of napropamide from the soil.

In general, all six alfalfa plants showed lower biomass under the exposure to napropamide. Also, napropamide had a negative effect on the chlorophyll content in leaves. Chlorophyll was shown to be sensitive to napropamide exposure. Previous study showed that most of organic xenobiotics triggered burst of reactive oxygen species (ROS) and consequently resulted in oxidative stress [21,32,33]. Superoxide (O_2^-) represents an instable species of reactive oxygen which can rapidly be converted to H_2O_2, and therefore, production of O_2^- by plants is usually accompanied by the appearance of H_2O_2 [23,34,35]. Overgeneration of ROS was detected in the alfalfa plants exposed to napropamide. The sensitive generation of $O_2^{-\bullet}$ and H_2O_2 can be used as a biomarker to illustrate the degree of oxidative stress.

Because high abundance of intracellular ROS directly causes oxidative damage to macromolecules such as biological membrane or DNA [10,23,33], we therefore determined the membrane lipid peroxides, expressed as thiobarbituric acid reactive substances (TBARS). However, treatment with napropamide appeared not to induce sufficiently a general damage to plasma membrane in the alfalfa plants, except for two cultivars in root and three in shoot, respectively. These results imply that some of the alfalfa genotypes have inherent tolerance to the pesticide. Therefore, these alfalfa cultivars can be potentially used for phytoremediation/phytodegradation of the pesticide-contaminated soil.

5. Conclusion

Napropamide residue as one of the organic xenobiotics affects the growth of six *M. sativa* genotypes at the toxic level (3 mg kg^{-1}). The plants differentially accumulated napropamide in tissues (root and shoot). However, there was no causal relationship between the level of accumulated napropamide and growth response in these plants. This suggests that they have different inherent mechanisms for tolerance to the pesticide. The differences were also reflected by the pattern of napropamide translocation and other biological parameters (*e.g.* chlorophyll content) and residues of napropamide in the rhizosphere. Although the cultivars appeared sensitive to napropamide-triggered generation of reactive oxygen species, not all of them showed significant oxidative injury to plasma membrane. The biological process can be interpreted as an internal tolerant mechanism in this species and may allow us to develop higher plant-based clearing-up materials for bioremediation of herbicide-contaminated soils.

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