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Regular Article

Root exudation of prometryn and its metabolites from Chinese celery (*Apium graveolens*)

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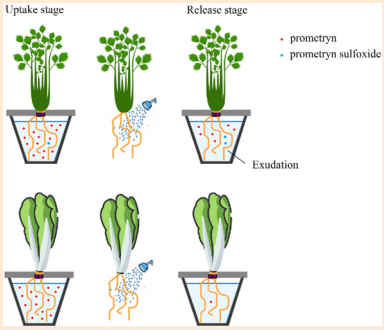
Supplementary material

Root exudates from Chinese celery (*Apium graveolens*) and Chinese cabbage (pak choi, *Brassica chinensis*) plants treated by prometryn, an herbicide, were qualitatively and quantitatively investigated and compared under hydroponic cultivation. Prometryn and its metabolites released into the nutrient solution were analyzed by ultra-performance liquid chromatograph coupled with orbitrap mass spectrometer to investigate whether this xylem-mobile herbicide is exuded from the roots. The results showed that celery and pak choi had different root exudation profiles. Celery metabolized prometryn to prometryn sulfoxide and released both compounds from the roots. In contrast, pak choi barely metabolized or actively released prometryn from the roots. The concentration of prometryn sulfoxide released from celery after 96 hr was 21 μg/L, which was nearly one-third that of released prometryn. Our results indicate that the root exudation and translocation of xylem-mobile herbicides could be significant in plants and are highly species dependent compared with phloem-mobile herbicides.



Keywords: prometryn, root exudation, plant metabolism, celery, hydroponic cultivation, xylem-mobile herbicide.

Introduction

Plants are capable of exuding certain exogenous substances, such as herbicides, from their roots.[1)] The exuded exogenous substances have been shown to affect microorganisms and plants in the area surrounding the roots.[2–4)] Furthermore, root exudation has been suggested to be involved in the environmental fate of herbicides in weed species and herbicide-resistant crops.[5–7)] However, data on the root exudation of herbicides are limited.

Several exogenous compounds can move freely and equilibrate rapidly between the phloem and xylem; however, owing to the considerably greater water flow in the xylem, only compounds retained in the sieve tubes of the phloem can be effectively transported to the roots and then released from there.[8,9)] Herbicides that can be transported in the phloem are considered to be affected by physicochemical properties, such as aqueous solubility, acid and base strength, hydrophilicity/lipophilicity, and molecular size. Among these, hydrophilicity and acidity are considered to be more decisive factors of translocation in plants by Grayson and Kleier,[10,11)] who developed a model for the phloem translocation of exogenous compounds. The model can make simple predictions of phloem mobility for a matrix of octanol/water partition coefficient (log K_{ow}) and ionization constant (pK_a) values. In the Kleier model, acidic herbicides with pK_a and log *K*ow values of <7 and <3, respectively, tend to remain in the phloem due to ion-trapping mechanisms, and this is consistent with the results of herbicides such as glyphosate,[3,5,7)] thifensulfuron-methyl,[12)] picloram,[13)] imazapyr,[14)] and quinclorac.[15)]

Prometryn (2,4-bis(isopropylamino)-6-methylthio-s-triazine) is a thiomethyl-s-triazine herbicide used for controlling annual grass and broadleaf weeds in modern agriculture. Due to its mean soil sorption coefficient (Koc) value of 400,[16)] prometryn has relatively low mobility in soil,[17)] and it is a common environmental pollutant in water and soils.[18)] According to the above-mentioned Kleier model, this non-ionized compound of intermediate lipophilicity (log *K*ow 3.34) was predicted to escape rapidly from the

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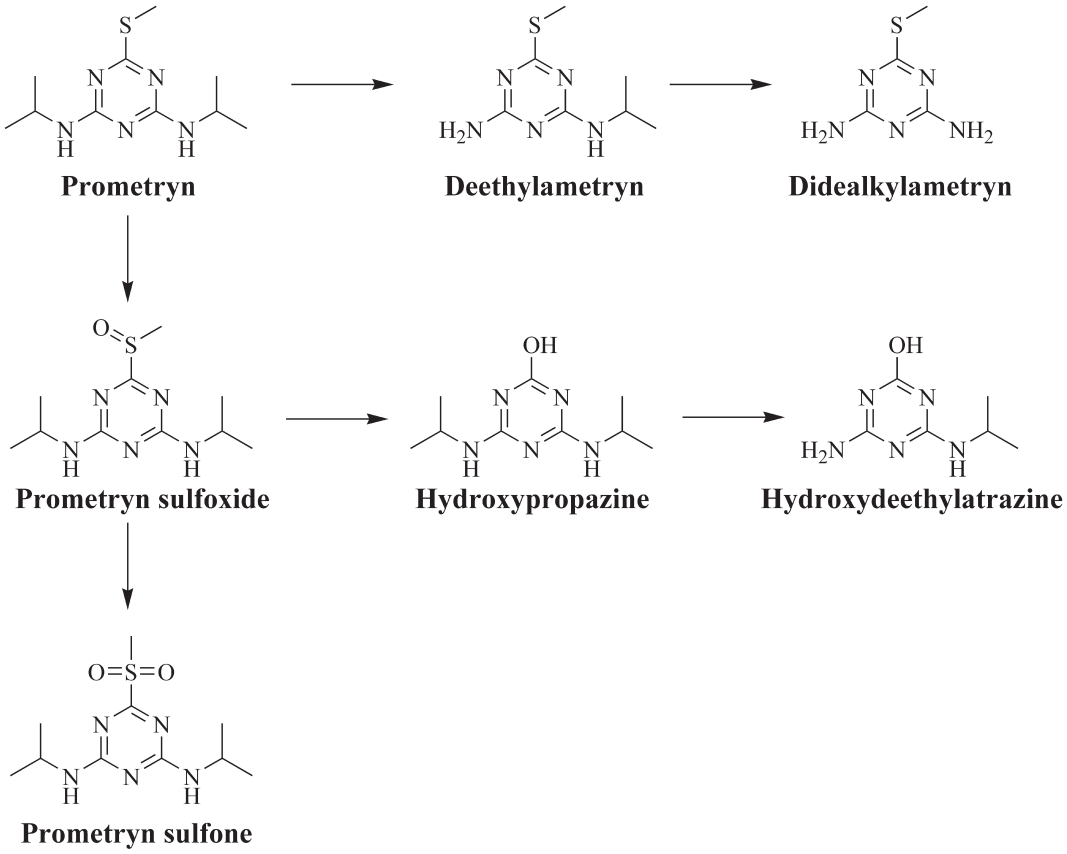

Fig. 1. Main metabolic pathway of prometryn in celery.

phloem and move predominantly to the xylem; specifically, it is considered to be a non-phloem mobile herbicide.[11)]

To the best of our knowledge, there are no data on the root exudates of xylem-mobile herbicides such as prometryn. The main purpose of this study was to investigate the occurrence and significance of the root exudation of prometryn and its metabolites from mature Chinese celery (*Apium graveolens* L.) and the Chinese cabbage, pak choi (*Brassica chinensis* L.), under hydroponic cultivation. Prometryn metabolism has been investigated in field-grown celery, and its metabolic pathway was reported by the U.S. Environmental Protection Agency in 1992.[19)] The main metabolites, which were the target compounds in this study, are shown in Fig. 1.

Materials and methods

1. Chemicals

Prometryn (99.90% w/w), hydroxypropazine (99.56% w/w), and hydroxydeethylatrazine (99.77% w/w) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Deethylametryn (99.9% w/w) and didealkylametryn (98.7% w/w) were purchased from TLC PharmaChem, Inc. (Ontario, Canada). Prometryn sulfoxide (98.0% w/w) and prometryn sulfone (96.0% w/w) were customized and synthesized by Shanghai Nafu Biotechnology Co., Ltd. (Shanghai, China). Acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (optima LC/MS) was supplied by Fisher Scientific (Shanghai, China). Ultrapure water was obtained from Sartorius Lab Instruments GmbH & Co. KG (Göttingen, Germany). All chemicals were of analytical reagent grade and were used without further purification. Modified Hoagland nutrient solution with 16 essential elements and N, P, K, Ca, and Mg concentrations of 115, 23, 185, 80, and 24 mg/L, respectively, was purchased from Fushan Yigeng Agricultural Technology (Yantai, Shandong, China).

2. Plant materials and growth conditions

Apium graveolens and *Brassica chinensis* plants used in the experiments were collected from Dalian, China. Plants were grown to maturity (4–4.5 months for celery and 30 days for pak choi) in a greenhouse in controlled conditions of 25°C/18°C under a 16 hr/8 hr day/night cycle. Air humidity was not controlled. Natural soil was obtained from plots that had not been sprayed with prometryn. Pots with mature plants were transferred to the laboratory and then gently removed from the soil. Their roots were rinsed with distilled water. After clearing the soil from the roots, the plants were acclimatized in modified Hoagland nutrient solution in a greenhouse at 20°C under a 16 hr/8 hr (light/dark) photoperiod for 2 weeks before the uptake experiment.

3. Uptake and release experiments and sample analysis

To observe the substances released from the roots of plants, prometryn was absorbed by the plants from the nutrient solution containing prometryn in the uptake experiment. Thereafter, the plants were transferred to a blank nutrient solution in the release experiment.

In the uptake experiment, plants of uniform size (approximately 100 g, fresh weight) were selected, and their roots were washed again with distilled water. The plants were placed in 1 L containers with 800 mL of modified Hoagland nutrient solution supplemented with 1.0 mg/L prometryn for 162 hr. The pH of the nutrient solution was measured at each sampling time point to ensure that it was within 6.0–6.5. Nutrient solution was periodically added to maintain the volume of 800 mL. Samples were collected from the nutrient solution, the plants were gently lifted, and the solution was stirred and mixed using a glass rod. The lower, middle, and upper layers of the nutrient solution were analyzed individually at 20–26 hr intervals for 162 hr. The results in the figures indicate the mean of three treatments and are expressed as the mean±standard deviation (mean±S.D.). To observe whether prometryn metabolites can be generated without plants, a contrast experiment was designed; it contained only the same amount of nutrient solution and prometryn.

In the release experiment, the roots were cleaned with dis-

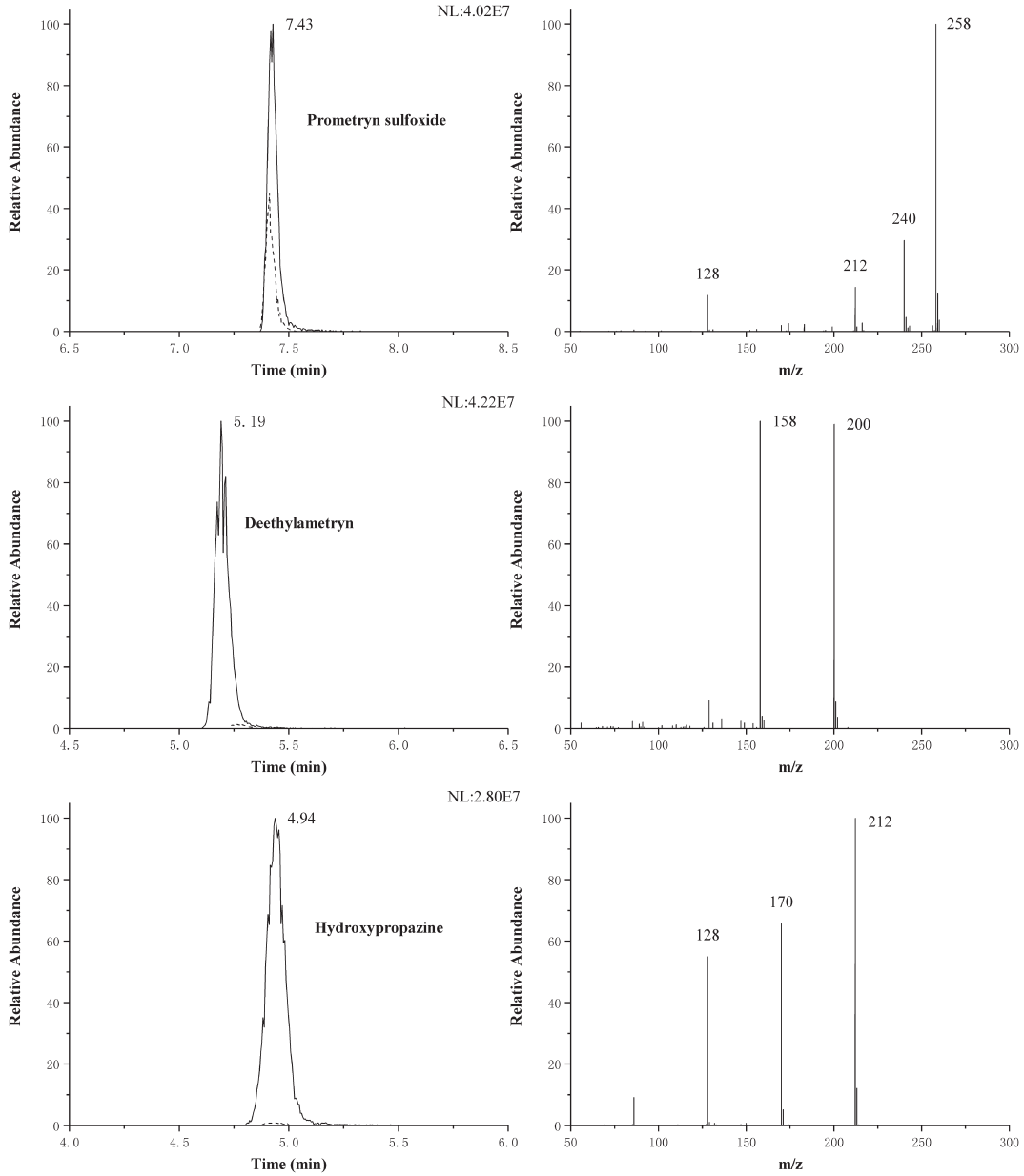

Fig. 2. Ion chromatograms and MS^2 spectra of prometryn metabolites spiked at 10 μg/L detected by UPLC-orbitrap-MS/MS (the dotted line represents the content of the compound detected in the uptake experiment with celery after 162 hr).

tilled water before the plants were transferred to a new container filled with 800 mL of nutrient solution without prometryn. Similar to the uptake experiment, samples were collected from the lower, middle, and upper nutrient solution layers and analyzed individually at 2–6 hr intervals for 96 hr.

In the uptake experiment, nutrient solution samples were diluted 10 times with the initial mobile phase (90% water and 10% acetonitrile with 0.10% formic acid) and then filtered through a 0.22 μm membrane filter and used for analysis. In the release experiment, nutrient solution samples were directly filtered and analyzed.

4. Preparation of standard solutions

The individual stock standard solutions of prometryn and its metabolites at 1,000 μg/mL were prepared in methanol by precisely weighing the solid standards and storing the solutions at −18°C. A mixed working standard solution was prepared in methanol containing prometryn at 100 μg/mL and other metabolites at 10 μg/mL, then stored at −4°C. The required matrix-matched calibration solutions were prepared by appropriately diluting the working standard solutions with blank nutrient solution and were analyzed immediately.

5. Instrumentation conditions

Chromatographic analysis of prometryn and its metabolites was performed using an Ultimate 3000 UPLC system (Thermo Fisher Scientific, Germering, Germany). Chromatographic separation was achieved on an Accucore aQ C18 column (150 mm×2.1 mm, 2.6 μm) from Thermo Fisher Scientific. The following linear-gradient program was applied, with the mobile phase (A) consisting of water with 0.1% formic acid and phase (B) consisting of acetonitrile: 0–4 min: 10–20% B; 4–5.5 min: 20–40% B; 5.5–10.5 min: 40–90% B; 10.5–12 min: 90–10% B. The flow rate was set to 0.30 mL/min, and the injection volume was 5 μL.

The UPLC system was coupled to a Q-Exactive Orbitrap MS (Thermo Fisher Scientific, Bremen, Germany), operated with a heated electrospray ionization (HESI) source in the positive mode. The ion-transfer capillary temperature, spray voltage, sheath gas flow rate, auxiliary gas flow rate, and S-lens RF level were set to 320°C, 3.6 kV, 40 arb, 10 arb, and 50 V, respectively. The Q-Exactive MS was tuned and calibrated in the positive mode once a week using calibration solutions.

Full MS-SIM and Full MS/dd-MS^2 (TopN) were used for quantitative and qualitative analyses. Full MS scan data were acquired at a resolution of 70,000 FWHM, with an Automatic Gain Control (AGC) target value of 1.0×10^6, a maximum ion injection time of 200 msec, and a scanning range of m/z 50–600. Data-dependent acquisition (dd-MS^2) parameters were used to obtain product ion spectra at a resolution of 17,500 FWHM with an AGC target value of 1.0×10^5 and a maximum ion injection time of 60 m sec. The normalized collision energy of each compound was set as 30%, 40%, and 50%, and the dynamic exclusion was set as 5 sec. A mass inclusion list including the precursor ion m/z of each analyte was used. Data were processed using Xcalibur™ version 2.1 (Thermo Fisher Scientific, Les Ulis, France).

6. Validation of the analytical methods

The method for analysis of prometryn and its metabolites was validated according to European Union guidelines.[20] For each compound, the limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the analysis of target compounds spiked in a blank nutrient solution that yielded S/N ratios of 3 and 10. The recovery and relative standard deviation (RSD) at low, medium, and high levels were calculated based on intraday analysis of standards spiked in blank nutrient solution (n=6).

Results and discussion

1. Identification of celery root exudates from prometryn

To identify major target analytes exuded into the nutrient solution from hydroponically cultivated celery plants, we detected prometryn and its metabolites released from long-term-cultivated plants (more than 6 months) using Q-Exactive Or-

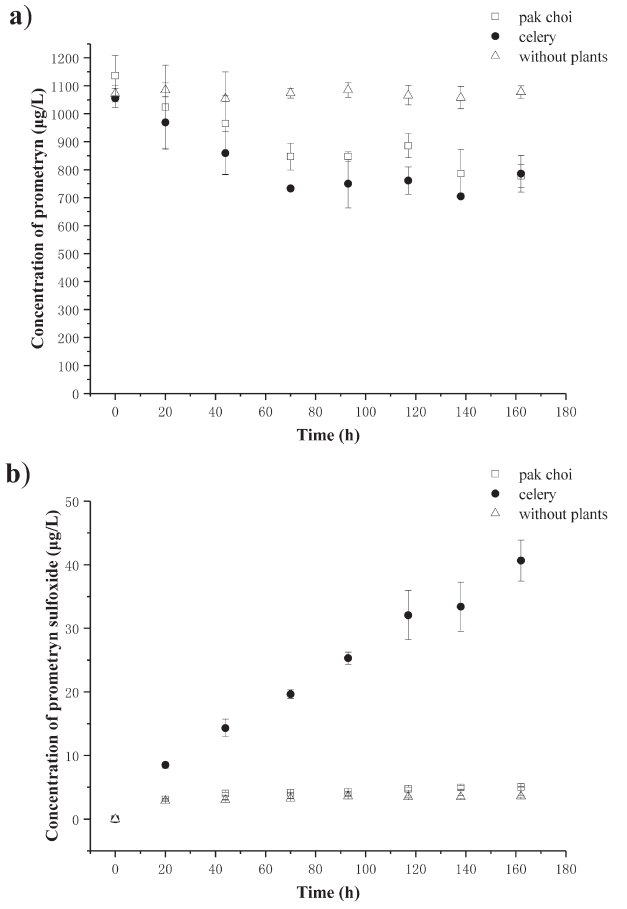

Fig. 3. Dynamic concentrations of prometryn (a) and prometryn sulfoxide (b) released from celery, pak choi, and under no plant conditions into hydroponic media during an uptake experiment over 162 hr (mean±S.D., n=3).

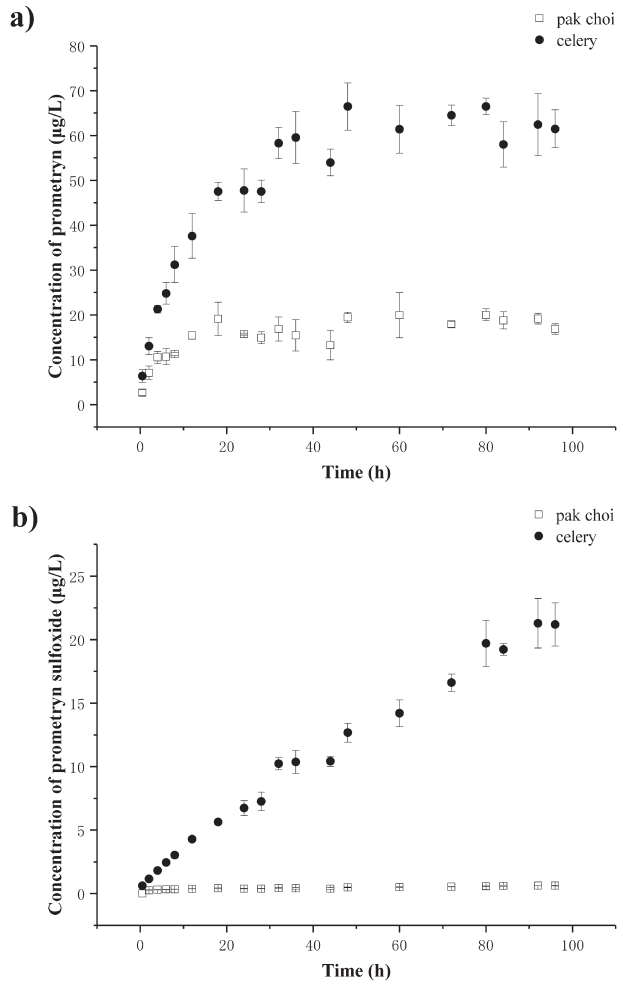

Fig. 4. Dynamic concentrations of prometryn (a) and prometryn sulfoxide (b) released from celery and pak choi into hydroponic media during a release experiment over 96 hr (mean±S.D., n=3).

bitrap MS in the full MS/dd-MS^2 mode (Supplemental Table S1). Prometryn and its metabolites were identified based on the exact masses of $[M+H]^+$ and MS^2 fragments and then confirmed using commercial standards and customized standards (prometryn sulfoxide and prometryn sulfone). All compounds except prometryn sulfone were detected in the sample. To observe whether prometryn sulfoxide is converted to prometryn sulfone upon further oxidation, prometryn sulfone was included as a target compound. To avoid byproducts of prometryn due to the hypochlorite present in tap water, we used ultrapure water to prepare the nutrient solutions.[21] We analyzed ultrapure water mixed with 1.0 mg/L prometryn for 48 hr; no prometryn sulfoxide was found in the ultrapure water during this period.

2. Method validation

Method validation data for the blank nutrient solution spiked with standards are summarized in Supplemental Table S2. The obtained calibration curves showed good linearity (>0.99). The LODs and LOQs were within 0.01–0.10 μg/L and 0.03–0.30 μg/L, respectively. The recoveries of the target compounds were within 78.0–108.6%, with RSDs ranging from 2.7 to 18.2%. According to European Union guidelines[20] on analytical quality control and validation procedures for analyzing pesticide residues in foods and feeds, the recovery should be within 70–120%, and RSDs should be <20%. All of our results met these criteria, indicating that the method used was appropriate for the determination of prometryn and its metabolites in a nutrient solution.

3. Uptake experiments

In the uptake experiments, prometryn was absorbed from the nutrient solution through the roots of plants of each species for 162 hr until equilibrium was reached. As shown in Fig. 2, only three metabolites or degradation products were detected in the celery uptake experiment after 162 hr, namely prometryn sulfoxide, deethylametryn, and hydroxypropazine; the amount of prometryn sulfoxide significantly increased, and the amounts of the remaining metabolites were negligible (<5.0 μg/L). Further oxidation of prometryn sulfoxide did not occur because prometryn sulfone was not detected. Meanwhile, negligible amounts of the three compounds were also generated in the contrast experiment (<5.0 μg/L); only the concentrations of prometryn and prometryn sulfoxide were considered in the uptake and release experiments. During the uptake experiment over 162 hr, the concentration of prometryn in the nutrient solution rapidly decreased during the first 70 hr for both plant species (Fig. 3a). After 70 hr, the concentration of prometryn fluctuated around 800 μg/L. At this stage, substances rapidly diffuse from the surrounding medium into the root and then slowly accumulate in the tissue.[22] Root absorption of prometryn by both species followed a similar pattern, represented by decreasing concentrations in the nutrient solution over the uptake period.

Besides prometryn, prometryn sulfoxide was a major metabolite in the celery root exudate (Fig. 3b). After 162 hr, the prometryn sulfoxide concentrations in the nutrient solutions of celery and pak choi were 46 and 6 μg/L, respectively. The cumulative prometryn sulfoxide concentration was significantly higher for celery than for pak choi throughout the experimental period, suggesting that metabolization and root exudation occurred continuously throughout the uptake experiment in celery.

4. Release experiments

To observe root exudates in the nutrient solution for the two species after the 162 hr uptake experiment, we rinsed the roots with sterile water five times to reduce residues on the root surface. The plants were then transferred to a new container filled with 800 mL of nutrient solution without prometryn to observe the exudates from the roots of both species. As shown in Fig. 4a, the cumulative prometryn concentration in the nutrient solution reached approximately 60 and 10 μg/L at 36 hr for celery and pak choi, respectively, and then more or less stabilized, indicating that prometryn was released from the roots of plants of both species and tended toward an equilibrium. The limited release of prometryn from the pak choi roots might have been passive, as

concentration gradients play a major role.[23] Three mechanisms mediate the root exudation of compounds: diffusion, vesicle transport, and ion channels.[24] However, celery released more prometryn into the cultivation medium than pak choi, which indicates that the root exudation of prometryn in celery is an active process, at least to a certain extent.

Similar to the findings in the uptake experiments, only for celery, prometryn metabolization and root exudation occurred continuously throughout the release experiments and did not show a tendency to slow down (Fig. 4b). It is noteworthy that after 96 hr, the concentration of prometryn sulfoxide released from the celery plants reached 21 μg/L, which was nearly one-third that of prometryn (61 μg/L after 96 hr). This is not a negligible amount, especially when considering that it is more toxic than prometryn.[21]

Notably, there is a gap between the amount by which prometryn decreased in the nutrient solution and the amount of prometryn that was released from plants. Hand *et al.*[25] determined that radiolabeled prometryn in the planted system closed this gap, and there was no significant uptake of radioactive residue by above-ground parts of the plants; specifically, prometryn dissipation occurred in the underground parts.

5. Exudation profiles of celery and pak choi

The release experiments revealed distinct prometryn root exudation profiles for celery and pak choi. For pak choi under hydroponic cultivation, active root exudation was hardly observed, which was anticipated, as prometryn is a xylem-mobile herbicide. However, for celery, although there are a few examples, the results of this study prove that the distinction between compounds mobile in the xylem and those mobile in the phloem is not clear. Nonetheless, xylem-mobile herbicides prometryn and prometryn sulfoxide can freely enter the symplast and be exuded from the plant roots. Although the mechanism remains unclear, it is highly likely that prometryn translocation and metabolization are species dependent, and the vascular structure of celery root may play a vital role.

Our results have the following implications. First, the translocation of herbicides depends not only on their physicochemical properties but also on the plant species, which has to be considered when applying herbicidal agents for weed control. Second, although soil cultivation was not conducted in our study, due to translocation and exudation in the hydroponic cultures, prometryn may end up in the soil root zone, where it can cause negative effects in non-prometryn-resistant plants *via* root uptake. Third, prometryn and its main metabolite, prometryn sulfoxide, may relocate from the root zone further into the environment, for example, into the groundwater. The effects of groundwater contamination by prometryn and its metabolites remain to be assessed.

Conclusions

In this study, prometryn, which is considered a xylem-mobile herbicide, was shown to be released from celery roots under hydroponic cultivation. Our results provide strong evidence that prometryn metabolization and translocation in plants are highly species dependent. Prometryn sulfoxide is a nonnegligible prometryn metabolite that may cause various negative effects in neighboring plants, and the effect of this new environmental contaminant should be assessed in the future.

Funding

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Electronic supplementary materials

The online version of this article contains supplementary material (Supplemental Table S1 and S2), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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